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ABSTRACT

This monograph contains 10 plant pathology experiments that were written to correspond to portions of a biology curriculum. Each experiment is suitable to a biology topic and designed to encourage exploration of those biological concepts being taught. Experiments include: (1) The Symptoms and Signs of Disease; (2) Koch's Postulates; (3) Monoculture and Disease Epidemics; (4) Plant Parasitic Nematodes from Soil; (5) Fungi from Soil; (6) Classification of Powdery Mildews; (7) Halo Blight of Bean; (8) Pectic Enzymes That Destroy Plant Cell Structure; (9) The First Virus Discovered--Tobacco Mosaic Virus; and (10) Plant Tumors Are Genetically Engineered by Agrobacterium tumefaciens. Background information and safety and waste disposal information, are also included. (WRM)



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*The National Association of Biology Teachers presents*

# LEARNING BIOLOGY WITH PLANT PATHOLOGY

*Written by Juliet E. Carroll*

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## CHAPTER 1 The Evolution of Plant Pathology from Biology

Plant pathology is an exciting, sometimes mysterious, and invariably multi-faceted field that relates directly to biology. The student may be fascinated to learn that plants can acquire diseases. When diseased, plants express symptoms that at first appear unusual until the inquisitive mind finds signs of the organism that is causing the disease. Accurate identification of the myriad of microorganisms that cause disease in plants is only one aspect of what plant pathologists do. Plant pathologists can potentially study every aspect of a disease. Some may work on conceptual theories about disease and what determines a plant's susceptibility to a plant pathogenic microorganism. From this research might come scientific facts about the genes that govern susceptibility in a plant. Research in plant pathology is diverse and includes many scientific disciplines. And because plant pathology contributes in many ways to society, careers in this field can be highly rewarding.

Biology provides the foundation for plant pathology. In plant pathology, the healthy versus diseased plant, its environment, the organisms that cause disease, and methods of disease management and treatment are studied. This encompasses several scientific disciplines—many of which branch out from biology. Knowledge of botany, horticulture, plant science, biochemistry, and molecular biology is necessary for the study of healthy and diseased plants. Again, molecular biology and biochemistry, but also mycology, microbiology, virology, and nematology are drawn upon when studying plant pathogens. Studying the environment in which the virulent pathogen and susceptible plant interact involves soil science, aerobiology, and meteorology. Finally, methods of predicting disease outbreaks and protecting plants must be developed; this involves mathematics, calculus, computer science, and chemistry. The diversity of plant pathology in terms of its relationship with a variety of scientific disciplines is analogous to human medicine and health-related fields.

As a result of the disease process, plants express many symptoms. The yield per acre of diseased crops is less than that of healthy crops. Entire fields of plants may be killed (Southern corn leaf blight in the 1970s) or desirable species eliminated from forests by diseases (chestnut blight in the early 1900s) (Figure 1.1). Some infections are relatively mild, causing slower growth or stunting of plants. Diseased plants may be unattractive in the landscape or market. The function of plants for human use may be altered or destroyed, as in a groundcover planting of pachysandra blighted and killed by Volutella blight.

The existence of people and society is dependent on the success of plant life on earth. Plants and products derived from them are used for food and drink, clothing, shelter, medicines, and animal feed, as well as for beautification and enjoyment. Plant

pathology evolved out of our need to protect plants, especially food plants from the scourge, of disease.

Figure 1.1. *Chestnut trees killed by the chestnut blight, circa 1910.*

The earliest botanists to study diseased plants did so because of severe epidemics that wiped out vast acres of food plants. To avoid famine, ways of protecting plants from disease were sought. Today economics tend to guide the study of plant diseases in order to maximize yield and dollar value of crops. Disease outbreaks can be predicted using meteorological data, monitored by scouting fields for symptoms and signs of disease, managed with resistant varieties and horticultural practices, and treated with fungicides and bactericides. How the pathogen interacts with the host in causing the diseased condition remains a central focus of plant pathology from describing key symptoms that aid in diagnosis through research on the molecular mechanisms of pathogenesis.

Plant pathologists like medical doctors can be general practitioners, teachers, researchers, and specialists. General practitioners include extension plant pathologists, diagnosticians, and private consultants. College and university professors may be responsible for teaching courses in plant pathology. Plant pathologists who conduct research may do so at a university, an agricultural experiment station, a private company, or a nonprofit organization. Specialists focus their knowledge on a specific crop area such as vegetables or on a particular group of pathogens such as viruses. The biology student who is aware of plant pathology may choose from the many employment opportunities that a career in this science can offer.

The plant pathology experiments in this monograph give a sampling of the many areas in this science that fit into a biology curriculum. Given a taste of plant pathology, more students might be recruited into this field and all students will develop a broader perspective of life on Earth. There is so much to learn from plant pathology, and these experiments will make this process exciting and fascinating.

## CHAPTER 2 Plant Diseases & Humanity

*Frances Brach Bergstrom*

Fossils dating back 400 million years give evidence that the first land plants were attacked by fungi and bacteria. Disease-causing microorganisms (i.e. pathogens) did not drive these primitive plants to extinction. Rather, plants and their pathogens evolved and continue to evolve. In natural plant communities, pathogens have reached a state of dynamic equilibrium with their hosts: both survive and reproduce. Once humans began to cultivate plants for food and other needs, though, this balance of coexistence often was upset. Humans unwittingly started cultivating the pathogens that affected the plants they found most useful! In the wild there are many factors that limit the effects of pathogens. In modern agriculture, plants of the same species with little or no genetic variability are grown in monoculture, increasing enormously the opportunities for infection and pathogen spread. Human activities affect plant diseases. Conversely, plant diseases have resulted in economic ruin, malnutrition, starvation, migration, and disease and death of people and livestock.

### *PLANT DISEASES AFFECT HUMANITY*

Immigrants have moved and continue to move plants to new locations, often far distant from where the plants originated. Sometimes native plants harbor a pathogen that will cause a more severe disease on the introduced crop than on its native host. An example of this is fireblight on apples and pears (Figure 2.1).

Immigrants to the United States brought apple and pear trees with them. The bacterium that causes fire blight is indigenous to the U.S. infecting various native plants in the rose family. It damaged its native hosts relatively little, but was found to be a serious threat to introduced apple and pear trees, making commercial pear growing, under certain conditions, impossible.

Figure 2.1. *Fireblight destroys a pear orchard in Colorado, 1914.*

Long distance introduction of pathogens occurs principally by the import of contaminated plants or plant products. The fungus that causes Verticillium wilt of alfalfa came to this country from Europe in infected seed. The fungus that

causes chestnut blight is thought to have been introduced into the U.S. on nursery stock from the Orient.

At the beginning of the 20th century, the American chestnut was the most important hardwood tree species in the eastern United States. It had great commercial value for tannin, lumber, pulpwood, poles, railroad ties, and edible nuts. In less than 50 years, the chestnut blight fungus destroyed practically every mature chestnut tree within its natural range, eliminating the American chestnut as a commercial species and nearly causing the tree's extinction. Since the fungus was not native to the U.S., natural selection had not produced any American chestnut trees resistant to infection.

The fungus that causes Dutch elm disease, along with the insect that transmits it, arrived in this country on elm logs imported from Europe to be used for veneer manufacture. Because of this devastating disease, American elm trees, prized as ornamental shade trees, no longer grace the streets of countless communities across the country. Soilborne pathogens can be transported not only on plants, but also on a farmer's boots, on farm equipment, and on vacationers' vehicles.

Famines are the most dramatic outcomes of crop disease epidemics. Famines have occurred when epidemics decimate a crop that a society depends on for their primary sustenance. The most famous of these is the Irish potato famine[1] (Figure 2.10). A fungal disease, potato late blight, destroyed the Irish potato crop in 1845 and 1846. Due to their singular dependence on potatoes, an estimated 1 million people died and 1 1/2 million people emigrated from Ireland.

Potato late blight struck many European countries and the United States at the same time that it struck Ireland. The disaster in Ireland was not repeated in those countries because those societies did not have a dietary dependence on potatoes. Moreover, late blight of potato also influenced history in 1917; it destroyed approximately one third of Germany's potato crop which comprised a large part of the wartime diet of the Germans. The reduction in the already scant food supply contributed to the breakdown in morale and physical endurance of the Germans at the end of World War I.

Other famines caused by plant diseases have taken a huge toll on human life. In 1733, 12,000 people on one Japanese island died because the rice crop failed. Rice stunt, a viral disease, is thought to have caused the crop failure. In 1942, brown spot, a fungal disease of rice, caused the great Bengal famine in which approximately 2 million people died.

Plant diseases have sometimes caused great suffering and death in ways other than by starvation. Mycotoxicoses are diseases of humans and animals caused by the consumption of foods and feeds infested with fungi that produce toxic substances (mycotoxins). The classic example of a mycotoxicosis is ergotism. Ergotism has

Figure 2.2. *The dark purple-gray sclerotia of ergot in a rye head.*

affected poor people in times of famine throughout the past 10 centuries.

Ergot is the survival structure of the fungus *Claviceps purpurea* and other *Claviceps* species that replaces the seed kernels of many grasses, especially rye. In most of Europe throughout the Middle Ages, rye was the main food cereal for many people. When harvests were bad, poor people did not separate the purplish black ergot from the rye since they needed all the grain they could get to mill into flour to make bread. Ergot (Figure 2.2) contains alkaloids that cause a variety of symptoms such as memory loss, blindness, double vision, confusion, delusions, hallucinations, muscle spasms, abortion, suppression of fertility, the cessation of lactation, gangrene, and, in very severe cases, death. In France the disease was known as the "Sacred Fire" because people who had contracted the disease had a sensation of being burnt.

Several epidemics have been recorded. One outbreak in Russia in 1722 following a very poor harvest allegedly kept Peter the Great from undertaking a campaign against Turkey. In 1771, the consumption of infected rye was pinpointed as the cause of ergotism. Outbreaks of the disease declined as grain was cleaned more carefully. However, in 1977 in the Wollo district of Ethiopia, an outbreak of ergotism was reported. Many villagers died. They had eaten barley mixed with seeds of wild oats that had been infected with ergot. Today ergotism is very rare in humans but continues to be of importance as an animal disease, causing loss of livestock from abortion and gangrene.

Figure 2.3. *Corn kernels with* Aspergillus *growing on them.*

Mycotoxins were responsible for the deaths of more than 1 million people in the Soviet Union in 1945. During World War II, much of the 1944 grain crop was not harvested. In the spring of 1945, people gleaned what they could from the fields even though the

grain was moldy with *Fusarium*. A mycotoxin produced by the fungus survived the baking process when the flour was made into bread, causing people to suffer necroses of the skin, hemorrhage, liver and kidney failure, and death.

Most mycotoxicoses are caused by common and widespread fungi such as *Aspergillus* (Figure 2.3), *Penicillium*, *Fusarium*, and *Stachybotrys*. For years, medical pathologists thought that *Fusarium* species found in cultures of specimens from cancer patients on chemotherapy were merely laboratory contaminants. Now the medical community is taking a much closer look at the possible relationship between the *Fusarium* species and the deaths of patients whose immune systems have been severely compromised by chemotherapy. The toxigenic *Fusarium* species, which are normally soilborne organisms, are able to grow in the bloodstream of these patients. Healthy people needn't worry about these common fungi. Their immune systems will resist them. Those most at risk of infection are AIDS patients and cancer patients whose immune systems are severely compromised.

Figure 2.4. *Defoliation of coffee plant by coffee rust. Plant on right is healthy.*

Plant diseases have sometimes been responsible for the demise of a thriving agricultural industry. When this happens, growers must search for an acceptable substitute crop that not only will grow well in that region, but also will be equally profitable. The growers then need to learn new techniques of producing the crop and find markets for the new crop. All this readjustment takes time and money, creating hardship for the growers and other people tied to the industry.

At one time, Ceylon (presently Sri Lanka) was one of the great coffee-producing countries of the world. Around 1867, coffee rust, a fungal disease, appeared in a coffee plantation in Ceylon (Figure 2.4). It quickly spread throughout Ceylon, and by 1871, coffee exports had dropped more than 50%. By 1893, coffee exports had dropped to less than 7% of those preceding the appearance of the disease. Coffee rust had destroyed the coffee industry in Ceylon. The growers and the Oriental Bank were ruined economically by this catastrophe.

As the years progressed, tea was planted in place of coffee. Tea drinking, so characteristic of the English people today, has been attributed to coffee rust. Up until the middle of the 19th century, the people of England drank coffee and tea in

somewhat equal amounts. Ceylon was part of the British Empire and supplied England with coffee. After coffee rust wiped out the coffee industry in Ceylon and other parts of the British Empire, Brazil became the main coffee-producing country in the world. Rather than shifting their market to Brazil, the English changed their beverage habits. By the 20th century, tea consumption overshadowed coffee consumption by a six-to-one ratio.

There are numerous examples of plant diseases that have caused losses worth millions of dollars and suffering to mankind. A study of the 1982 world crop production found that, in general, diseases destroyed approximately 12% of the crop before harvest, insects another 12%, and weeds another 10%. Postharvest losses to pests including plant pathogens ranged from 9 to 20%. Losses to pests tended to be much greater in underdeveloped nations (where an average of 57.6% of the population was engaged in agriculture) than they were in the developed ones (where an average of only 11.6% of the population was engaged in agriculture). The impact is even greater when one considers that developing nations have a much greater population than the developed nations and produce relatively less food and fiber.

### *HUMANITY COPES WITH PLANT DISEASE*

How has humanity coped with plant disease? Up until the 1800s, the causes of plant diseases were a mystery. The destruction caused by diseases made people fearful, so they turned to their gods. Every spring the ancient Romans held a festival, the Robigalia, to appease the god Robigus, who was thought to have the power to ward off wheat rust. Prayers and sacrifices of a sheep and a reddish brown dog, the latter the same color as the rust fungus to be averted, were offered to Robigus.

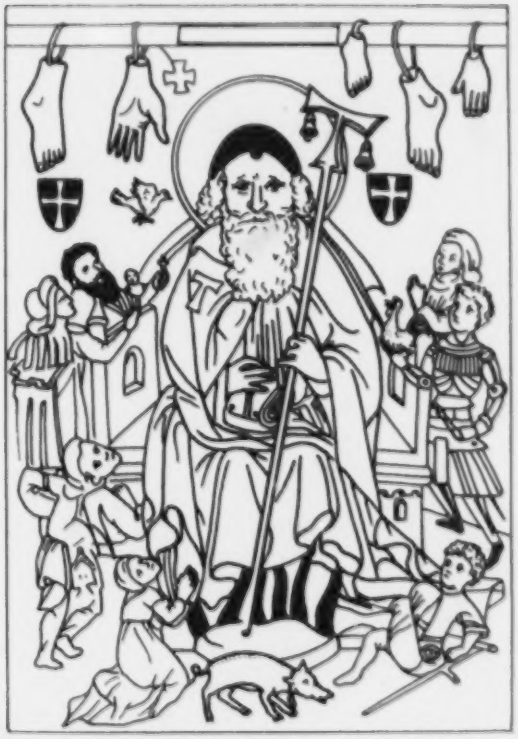

Figure 2.5. *St. Anthony. Redrawn from a 1215 A.D. woodcut made in Germany.*

In the Old Testament of the Bible, Moses warned the Israelites that if they didn't obey Yahweh's commandments, Yahweh would send curses on them. The people would not only be struck down with diseases, but their crops would be struck down by drought, blight, and mildew (Deuteronomy 28: 23). Leviticus 14: 33-57 addressed the problem of "leprosy" of houses, characterized by a

reddish or greenish pitting of the walls. The fungus responsible for the leprosy caused dry rot of the timbers. The priest was to be called in to direct the people in ridding the house of the leprosy and to offer sacrifices as a rite of atonement for the sin of the house, thereby making the house "clean."

Figure 2.6. *Fairy ring growing in a lawn.*

In the 12th century ergotism was associated with St. Anthony, a Christian monk who was believed to have power over fire (Figure 2.5). Many people who suffered from the painful disease of ergotism which they called the "Sacred Fire," made pilgrimages to the church where St. Anthony's bones were kept. Many miraculous cures were claimed.

In Europe, many innocent people were persecuted for witchcraft during the 17th and 18th centuries. The incidence of witchcraft trials followed a pattern: They took place in areas dependent on rye as a staple food where weather conditions favored ergot. It has been proposed that the symptoms of bewitchment suffered by both people and animals were actually caused by ergot alkaloids.

Superstitions about plant diseases abounded. Fairy ring disease of turfgrass was attributed in England to fairies dancing round and round and in Germany to the dancing of witches (Figure 2.6). In the Tyrol (a region in the Alps of western Austria and northern Italy), people believed that a winged dragon flew over the fields, scorching the grass with his tail, causing the fairy rings. Fairy rings are caused by the outward growth of cer-

Figure 2.7. *Witches'-broom of willow, cause unknown.*

tain mushrooms, most notably *Marasmius oreades*, from the point at which they first became established. Mushrooms grow in a browned, almost bare ring of grass around this point. Rings of dark green grass border the brown ring.
Witches were also associated with growths on trees that consisted of a mass of small twigs growing upward in a tight cluster that somewhat resembled a broom. These growths were thought to be caused by witches flying overhead on their broomsticks, hence the name, witches'-brooms (Figure 2.7). The witches'-brooms were probably caused by certain fungi that stimulate abnormal and excessive branching, although other pathogens also cause witches'-brooms.

Mistletoes are parasitic plants living on trees (Figure 2.8). According to Greek, Norse and Germanic legends, mistletoe was a plant vested with supernatural powers for good and evil. The Druids used it as a sacred emblem in their religious rites. Herbalists of the early Christian era claimed that mistletoe was once a forest tree but became dwarfed out of shame when its wood was used to make the cross upon which Jesus Christ died. Amulets made of the plant were often worn to ward off diseases. It was also used as an antidote for poisons and to cure epilepsy. Today it is used as a Christmas decoration. A person standing beneath it hopes to be kissed.

Figure 2.8. *Mistletoes growing in a deciduous tree.*

We now know that plant diseases are caused by fungi, bacteria, mycoplasma-like-organisms, parasitic higher plants, viruses, viroids, nematodes, and protozoa, as well as abiotic causes such as nutrient deficiencies and air pollution. They are influenced by a multitude of factors, and so an integrated approach must be taken to control them.

Crops are bred for disease resistance. In cases where seedborne pathogens cause heavy losses, growers buy seed that has been grown under special conditions to exclude the pathogen. Seeds, tubers and other plant propagative materials can be tested for the presence of pathogens and can be treated with chemicals and physical treatments to reduce or eliminate pathogen contamination.

Farm practices are very important in disease control. Sometimes a farmer can control a plant pathogen effectively by controlling the insects and weeds that play a part in the disease cycle. The use of certain types of irrigation and cultivation practices helps

reduce the spread of plant pathogens. Crop rotation (planting crops not attacked by the particular pathogen for three or four years) allows for the decomposition of crop residues which provide a substrate for many plant pathogens to survive. Proper soil fertility can help minimize disease losses.

Pesticides that are applied at the proper time and at the recommended rates are very effective in controlling diseases. When pesticides are used properly, there is little risk of damage to the environment or of residues posing a threat to human health. Government regulations allow residue levels on the crop at harvest that are but a tiny fraction of the smallest amount that causes maladies in laboratory animals.

Control measures must be used judiciously. Continual use of one pesticide, one resistant plant variety, or one biological control measure can lead to the development of new races of the pathogen that are resistant to the control measure. Plant pathogens can cause considerable losses in stored produce and grains, so storage facilities are designed to produce conditions least favorable to disease development.

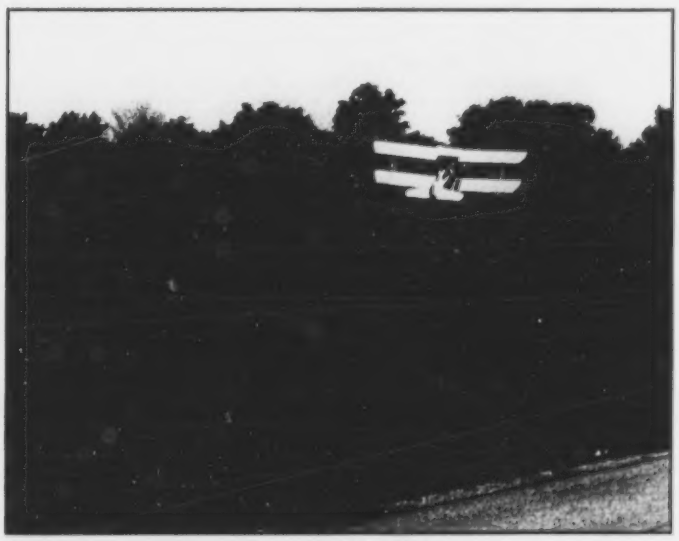

Figure 2.9. *Aerial application of fungicides.*

Extreme measures must be taken at times to control plant diseases. After World War I, the U.S. government set up an immense and costly eradication program to destroy barberry bushes (the alternate host of stem rust of wheat) in an effort to control wheat rust. This program was thought to be successful in decreasing the severity of epidemics. Moreover, another successful eradication program was undertaken to remove *Ribes* species (gooseberries and currants, the alternate hosts of white pine blister rust) from white pine forest areas in an effort to control white pine blister rust.

Quarantines are set up to prevent the introduction of pathogens into new areas. This may be on a local, state, federal or international basis. Although costly, it is believed that the money spent on successful quarantines is but a fraction of the economic losses that would occur if they weren't in place.

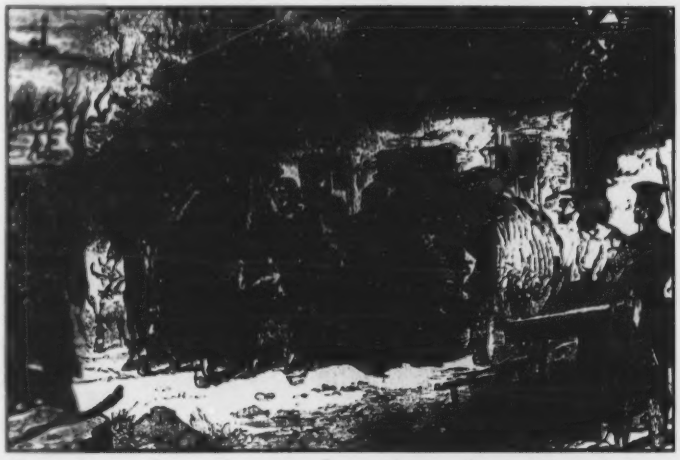

Figure 2.10. *An eviction; tenants are dragged out with the help of troops.*

When an epidemic is imminent, quick action and teamwork by people in diverse fields is needed to avoid catastrophe. An example of this is the response to the wheat leaf rust epidemic in northwestern Mexico in 1977. A series of unusual events[2] led to the epidemic that could have had catastrophic results. However, government scientists presented the problem to the Mexican authorities along with the recommendation to use two new systemic fungicides to try to reduce the impact of the epidemic. The government acted quickly in enacting a government- sponsored program of aerial application of the two new fungicides (Figure 2.9) that were shipped from Germany, Columbia and Italy. The Mexican government waived duties and facilitated import licenses. Military planes were used to fly the fungicides to the distribution points within the Sonora State.

In addition, 91 technicians assessed the situation in wheat fields and determined priorities for spray application. Two hundred-forty field supervisors handled the logistics of fungicide and aircraft allocation to each zone and supervised the spray applications. One hundred pilots made their aircrafts available for three consecutive weeks. The banking system provided immediate credit to cover labor, chemical and freight expenses. The farmers and farmers' organizations recognized the emergency and accepted all technical decisions relating to the epidemic. Fields were given one or two aerial applications of fungicide. With the spray program, nearly normal yields were obtained that year in the Sonora State in contrast to more than 40% losses experienced in an area south of the region.

Plant pathology is the branch of science that seeks to understand the causes and mechanisms of plant diseases and strategies to manage them. Plant diseases, often exacerbated by human activity, have had major impacts on humanity. Application of the findings of plant pathology research will allow people to be better stewards of plants and the environment.

***Footnotes***

1. The poorest people in Ireland, the cottiers, lived almost exclusively on potatoes, consuming from 8 to 14 pounds of potatoes per day. They grew their potatoes on a

quarter acre plot of land that they rented. In those days, estates were subdivided many times over, the land owner as well as the middlemen all getting their share of rent money. The cottiers, at the bottom of the rent pyramid, paid more than 60% of their annual income on rent. Left with so little, they frequently found themselves in debt. The Irish resisted efforts to improve their lives by restructuring the agricultural system and adopting new methods of agriculture because they feared the British would cheat them of their gains. They feared the loss of their independence. Potato late blight, a fungal disease, destroyed the potato crops in 1845 and 1846. Some 4 1/2 million cottiers and poor farmers faced starvation. There was food in the country, perhaps enough to have nourished the entire population for a year, but the poor people had no money to buy it. A relief commission was set up and maize was imported from America. The British did not feel it prudent to simply give the maize away to the starving Irish. They required that the Irish work for their ration of "chickens' food". Road-building projects were set up throughout Ireland. Many roads went from nowhere to nowhere. To ensure that only those in dire straits would work the roads, the British set up relief centers a mile or more away from the villages they served. At these centers, corn meal was made into an unpalatable wet mash that could not be stored or resold on the market. Every day people traveled to get their portion of mash. Those who were sick either died on the way to the relief center or shared other people's portions. Although the 1847 potato crop was good, those unable to pay rent were evicted from their homes (Figure 2.10). Between 1845 and 1860, an estimated 1 million people died, and 1 1/2 million people emigrated from Ireland as a result of the famine. It was said that the potato blight brought about more death and suffering than any other disaster since the Napoleonic Wars. It also brought about the change of the policy of protectionism, opening the British market to free trade.

2. Wheat growers in the Sonora State quickly adopted the newest, best yielding, rust resistant wheat variety. Thus, the majority of the wheat acreage was planted in this one variety. Within two years, races of the wheat rust fungus evolved that overcame this resistance. Normally, wheat was planted from mid-November to mid-December and harvested in May. Crop residues were burned and the land was prepared for a summer crop of soybeans. However, four years of drought drastically reduced the amount of irrigation water available for the soybean crop in 1976. This, plus land tenure problems, led to the majority of the land being left fallow. In these fallow fields, many volunteer wheat plants grew from seed left after harvest and became infected with rust. Wheat planting dates were influenced drastically in 1976 by fate and man. Hurricane Liza brought rain to a small area of the region in early October. Some farmers, having no summer crops in, took this opportunity to plant wheat early and thus benefit from the soil moisture provided by Liza. These wheat plants were infected with rust spores from volunteer plants. Farmers in the rest of the region

planted wheat from mid-November through January as land tenure problems were settled. Thus, planting was staggered and delayed up to one month beyond the normal planting season. Weather conditions progressed from adequate to ideal for the rust fungus to propagate. The stage was set for a wheat leaf rust epidemic of catastrophic proportions.

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Findlay, W. P. K. 1982. *Fungi: Folklore, Fiction, and Fact.* Eureka, CA: Mad River Press. 112 pp.

Large, E. C. 1940. *The Advance of the Fungi.* New York: Henry Holt and Co. 480 pp.

Schumann, G. 1991. *Plant Diseases: Their Biology and Social Impact.* St. Paul, MN: APS Press. 397 pp.

Stefferud, A. ( Ed.). 1953. *Plant Diseases: The Yearbook of Agriculture.* Washington, DC: USDA, US Superintendent of Documents. 940 pp.

## CHAPTER 3 About the Monograph

This monograph contains 10 plant pathology experiments that have been written to correspond to portions of a biology curriculum. Each experiment is suitable to a biology topic and is designed to encourage exploration of concepts being taught about that topic as follows:

| Title of Experiment | Biology Topic |
|---|---|
| The Symptoms & Signs of Disease | Plants |
| Koch's Postulates | Microbial Disease |
| Monoculture & Disease Epidemics | Ecology |
| Plant Parasitic Nematodes from Soil | Invertebrates |
| Fungi from Soil | Microbiology, Fungi |
| Classification of the Powdery Mildews | Microbiology, Fungi |
| Halo Blight of Bean | Microbiology, Bacteria |
| Pectic Enzymes Destroy Plant Cells | Cell Structure |
| The First Virus Discovered - Tobacco Mosaic Virus | Microbiology, Viruses |
| Plant Tumors Are Genetically Engineered by *Agrobacterium tumefaciens* | Genetics |

Below are suggestions for the application of each of the experiments:

The exercise on symptoms and signs of disease will acquaint students with the plant diseases indigenous to their area. This exercise can also be used in the study of microbial disease and will be an excellent way to sharpen observational skills.

The exercise on Koch's postulates could be used to illustrate the importance of methodical scientific procedures to prove the involvement of an organism with a disease. By fulfilling Koch's postulates, students will gain an appreciation of the importance of obtaining unequivocal proof that a specific organism is the cause of a disease.

The exercise on monoculture and disease epidemics could be used during discussions of the biology of natural ecosystems. The difference in disease progress in a genetically diverse population (natural ecosystem) versus that in a monocultural system containing genetically identical individuals could be demonstrated.

The exercise on plant parasitic nematodes from soil could be utilized during discussions of invertebrates. Plant parasitic nematodes could be used to demonstrate the characteristics of this group. Extraction of plant parasitic nematodes from soil would give students the opportunity to experience invertebrates in a real situation. In addition, the exercise would illustrate that the invertebrates (including nematodes)

are just one of several different groups of organisms which are present in natural soil.

The exercise on fungi from soil could be used to demonstrate the diversity of organisms in natural soil and the differences in microflora found in different soils. Also, the technique used to isolate the fungi will demonstrate the principal of dilution plating to recover microbes in pure culture from a source rich in microbial life. In addition, this experiment could be used during discussions on the classification of fungi and fungal morphology.

The exercise on powdery mildews would be useful when studying the classification of fungi and would serve to demonstrate the diversity which exists in fungi. Because the materials for this exercise are readily available in nature, discussion(s) of the types of reproduction (i.e. sexual vs asexual) in fungi would be facilitated.

The exercise on halo blight of bean could be used to illustrate the characteristics of a bacterial disease of plants while learning the techniques used to grow bacteria in pure culture. The interesting phenomenon of fluorescent pigment production by *Pseudomonas* spp. would be demonstrated. This exercise also would demonstrate the involvement of a toxin in disease, which could serve as a basis for discussions regarding the importance of toxins in microbial disease. The use of a microbial assay for detection of toxin would provide an example of one of the tests commonly used in toxicology.

The experiment on pectic enzymes that destroy plant cell structure could be incorporated into the section of biology that examines the components, structure and function of cell walls. Following a discussion and examination of healthy plant cell walls, the effects of pectolytic enzymes produced by a plant pathogen on plant tissue could be observed.

The exercise on tobacco mosaic virus could be used during discussions related to the characteristics of viruses. In addition, tobacco mosaic virus could be used to exemplify structure of proteins and nucleic acids, and the relationships between these two types of molecules.

The experiment, plant tumors mediated by *Agrobacterium tumefaciens*, will support studies of genetics using the Ti plasmid as an example of genetic control in the expression of disease symptoms. Since the disease disrupts normal cellular growth and division, this exercise would foster discussions of normal cell division.

References are listed for each experiment and serve two functions. The first is to provide an overview of the topic and the second is to provide examples of research

on the topic. Experiments can be completed without reading the references. However, every high school should have in its library a copy of *Plant Pathology* (3rd ed. (1988, by G. N. Agrios). This book is comprehensive and written in language that could be readily understood by biology teachers interested in this field. There is a short section from this book cited under each experiment. Students may decide to investigate plant pathology further for independent study or for science fair competition. The research references may provide ideas for such investigation, even though these are written for the scientific community rather than the student and teacher.

The chapters preceding the experiments give detailed information on supplies and techniques. Chapter 4 covers safety with laboratory chemicals. Become familiar with the information here and refer to specific information before proceeding with an experiment. Chapter 5 gives information on obtaining pathogens from American Type Culture Collection, Carolina Biological Supply Company, and Ward's Natural Science Establishment. Chapter 5 also contains important information on how to dispose of infected plants and/or pathogens. When working with native collections as in Classification of Powdery Mildews, Fungi from Soil, The Symptoms and Signs of Disease, and Plant Parasitic Nematodes from Soil, the materials may be disposed of as is other waste. When working with the other experiments the guidelines for the disposal of pathogens and infected plants in Chapter 5 must be followed. In Chapter 6 recipes for media used to culture the microorganisms are given. The phrase "using sterile technique" in some experiments will refer to the explanations of culturing in Chapter 7. This chapter has information on how to prepare and maintain pure cultures of bacteria and fungi.

When additional help is needed, contact your local university, college, or experiment station. Questions that arise may also be directed to the American Phytopathological Society, Youth Programs Committee, 3340 Pilot Knob Road, St. Paul, MN 55121-2097.

The plants selected for these experiments will grow well on a windowsill or under lights. When working with tomato plants there is the option of purchasing young plants in the spring before the gardening season from a nursery, greenhouse, or garden center. Alternatively, a local greenhouse or nursery might be willing to grow tomato plants for the class. All plants should be grown in sterile potting mix (such as Fafard mix or Peatlite mix) and fertilized with an appropriate fertilizer (such as Rapid Gro™) according to manufacturer's directions. Avoid growing plants near hot or cold air vents. Remember that plants do not bleed, wiggle, smell of formalin or some other preservative, and so often are not as offensive for students to probe, dissect, examine, or inoculate. For high school and introductory college students, plant pathology offers a world of experimental inquiry that is unavailable in vertebrate biology.

***References***

*Textbooks:*

Agrios, G. N. 1988. *Plant Pathology, 3rd ed.* New York: Academic Press. 803 pp.

Kenaga, C. B. 1986. *Principals of Plant Pathology, 2nd ed.* Prospect Heights, IL: Waveland Press, Inc. 402 pp.

Schumann, G. 1991. *Plant Diseases: Their Biology and Social Impact.* St. Paul, MN: APS Press. 397 pp.

Stefferud, A. (Ed.). 1953. *Plant Diseases: The Yearbook of Agriculture.* Washington, DC: USDA, US Superintendent of Documents. 940 pp.

*Laboratory Guides:*

Baudoin, A. B. A. M. (Ed.). 1988. *Laboratory Exercises in Plant Pathology: An Instructional Kit.* St. Paul, MN: APS Press. 196 pp.

Boothroyd, C. W. & Kelman, A. 1966. Laboratory Experiments in Plant Pathology. *The American Biology Teacher, 28*:478-491.

Dhingra, O. D. & Sinclair, J. B. 1985. *Basic Plant Pathology Methods.* Boca Raton, FL: CRC Press, Inc. 355 pp.

Kenaga, C. B., et al. 1986. *Plant Disease Syllabus.* West Lafayette, IN: Balt Publishers. 224 pp.

Tuite, J. 1989. *Laboratory Exercises and Methods in Plant Pathology - Fungi and Bacteria.* West Lafayette, IN: Purdue University. 205 pp.

Zuckerman, B. M., et al. (Eds.). 1990. *Plant Nematology Laboratory Manual.* Amherst, MA: The University of Massachusetts Agricultural Experimental Station. 252 pp.

*Videotapes:*

Haning, B. & Campbell, C. L. 1989. *Healthy Plants - Our Future.* St. Paul, MN: APS Press. 21 min.

# CHAPTER 4 Laboratory Chemical Safety & Precautions

***Chemical Safety Data***

Key hazard words are used in this chapter to describe the hazard associated with a particular chemical as follows:

**CAUTION** - relatively nonhazardous to slightly hazardous.

**WARNING** - moderately hazardous.

**DANGER** - hazardous to highly hazardous.

Information about chemicals that may pose a hazard is given below and also in the relevant experiments. Always follow the precautions on the manufacturer's label. All solutions should be prepared by the teacher or under the teacher's supervision. Most of the chemicals used are more hazardous in solid or concentrated form than in solution. Right to Know laws require that the information contained in the Material Safety Data Sheet (MSDS) for a particular chemical be available to the user of that chemical. This chapter and the information in it are not meant to substitute for the MSDS sheets.

*Acetic acid - **DANGER** Flammable. Avoid all sources of ignition. Avoid inhalation. Use solutions in fume hood. Avoid contact with skin and eyes. Wear gloves and goggles.*

*Ammonium chloride ($NH_4Cl$) - **WARNING** Do not ingest. Avoid contact with eyes. Wear goggles and gloves.*

*Bleach (sodium hypochlorite) - **DANGER** Avoid contact with skin, eyes and clothing. Wear gloves, goggles, and lab coat.*

*Calcium chloride ($CaCl_2$) - **WARNING** Avoid contact with skin and eyes. Wear gloves and goggles. Do not ingest.*

*Carborundum - **CAUTION** Avoid inhalation of dust. Avoid contact with eyes and skin.*

*Celite - **CAUTION** Avoid inhalation of dust. Avoid contact with eyes and skin.*

*Ethanol - **DANGER** Highly flammable. Avoid all sources of ignition.*

*Glycerol - **CAUTION** Mix only as directed.*

*Magnesium sulfate ($MgSO_4 \cdot 7H_2O$) - **WARNING** Avoid contact with skin and eyes. Wear gloves and goggles. Do not inhale or ingest.*

*Potassium phosphate dibasic ($K_2HPO_4$) - **WARNING** Avoid contact with skin and eyes. Wear gloves and goggles.*

*Potassium phosphate monobasic ($KH_2PO_4$) - **CAUTION** Avoid contact with skin and eyes. Wear gloves and goggles.*

*Sodium acetate - **WARNING** Avoid contact with skin and eyes. Wear gloves and goggles. Use solutions in fume hood.*

*Sodium phosphate dibasic, anhydrous ($Na_2HPO_4$) - **DANGER** Avoid contact with eyes, skin and clothing. Wear gloves, goggles, and lab coat. Do not inhale dust. Wear mask when handling solid. Wash thoroughly after handling.*

*Streptomycin sulfate - **CAUTION** Do not ingest.*

*Thiabendazole - **CAUTION** Wear gloves when handling. Do not ingest or inhale.*

***References***

Committee on Hazardous Substances in the Laboratory, Assembly of Mathematical and Physical Sciences, and National Research Council. 1981. *Prudent Practices for Handling Hazardous Chemicals in Laboratories*. Washington, DC: National Academy Press. 291 pp.

Committee on Hazardous Substances in the Laboratory, Assembly of Mathematical and Physical Sciences, and National Research Council. 1983. *Prudent Practices for Disposal of Chemicals from Laboratories*. Washington, DC: National Academy Press. 282 pp.

# CHAPTER 5 Obtaining & Disposing of Pathogens & Plants

*OBTAINING PLANT PATHOGENS*

All the plant pathogens used in these experiments are available from the American Type Culture Collection (ATCC). Many of the microorganisms are also available from Carolina Biological Supply Company and Ward's Natural Science Establishment, Inc.

**American Type Culture Collection** has an account set up for all high school orders. To place an order, send a purchase order number with your request to:

American Type Culture Collection
c/o Sales and Marketing Department
12301 Parklawn Drive
Rockville, MD 20852

For toll-free telephone orders call 1-800-638-6597, Monday thru Friday, 8:30 am to 5:30 pm EST from anywhere in the continental USA. From Maryland, call collect 0-301-881-2600. When placing orders include the following:

- purchase order number
- shipping and billing address
- ATCC catalogue numbers
- name of person who signed the compliance form
- any necessary permits - supplied on request by ATCC.

The following microorganisms are available from ATCC:

| Microorganism | Catalogue No. |
|---|---|
| *Erwinia carotovora** | 15713 |
| *Agrobacterium tumefaciens* strain A348 (wild type) | 51317 |
| *Agrobacterium tumefaciens* strain C58 (heat curable) | 33970 |
| *Agrobacterium tumefaciens* strain A136 (cured) | 51350 |
| *Agrobacterium rhizogenes* | 15834 |
| Tobacco mosaic virus | PV 135 |
| *Pseudomonas syringae* pv. *phaseolicola** | 19304 |
| *Escherichia coli* strain K-12 | 10798 |
| *Escherichia coli* derivative of strain K-12 | 14948 |
| *Escherichia coli* strain K-12 | 23716 |
| *Escherichia coli* strain K-12 | 25404 |
| *Fusarium oxysporum* f.sp. *lycopersici* | 16322 |
| *Rhizoctonia solani** | 52185 |

* Require a USDA PPQ 526 permit accompanying order. A copy is included in this chapter (see p. 24). Information on completing this form is available from the Sales Department at ATCC or from USDA, APHIS, Plant Protection and Quarantine, Biological Assessment Support Staff, Hyattsville, MD 20782.

No permit can be issued to move live plant pests or noxious weeds until an application is received (7 CFR 330 *(live plant pests)* or 7 CFR 360 *(noxious weeds)*).

FORM APPROVED - OMB NO. 0579-0084

U.S. DEPARTMENT OF AGRICULTURE
ANIMAL AND PLANT HEALTH INSPECTION SERVICE
PLANT PROTECTION AND QUARANTINE
BIOLOGICAL ASSESSMENT SUPPORT STAFF
HYATTSVILLE, MARYLAND 20782

# APPLICATION AND PERMIT TO MOVE LIVE PLANT PESTS AND NOXIOUS WEEDS

**SECTION A - TO BE COMPLETED BY THE APPLICANT**

1. NAME, TITLE, AND ADDRESS *(Include Zip Code)*

2. TELEPHONE NO.

3. TYPE OF PEST TO BE MOVED
☐ Arthropods ☐ Noxious Weeds
☐ Pathogens ☐ Other *(Specify)*

| A. SCIENTIFIC NAMES OF PESTS TO BE MOVED | B. CLASSIFICATION (Orders, Families, Races or Strains) | C. LIFE STAGES IF APPLICABLE | D. NUMBER OF SPECIMENS OR UNITS | E. SHIPPED FROM (Country or State) | F. ARE PESTS ESTABLISHED IN U.S. | G. MAJOR HOST(S) OF THE PEST |
|---|---|---|---|---|---|---|
| 4. | | | | | | |
| 5. | | | | | | |
| 6. | | | | | | |

7. WHAT HOST MATERIALS WILL ACCOMPANY WHICH PESTS *(Indicate by line number)*

8. DESTINATION | 9. PORT OF ARRIVAL | 10. APPROXIMATE DATE OF ARRIVAL OR INTERSTATE MOVEMENT

11. NO. OF SHIPMENTS | 12. SUPPLIER | 13. METHOD OF SHIPMENT ☐ Air Mail ☐ Air Freight ☐ Baggage ☐ Auto

14. INTENDED USE *(Be specific, attach outline of intended research)*

15. METHODS TO BE USED TO PREVENT PLANT PEST ESCAPE | 16. METHOD OF FINAL DISPOSITION

17. *Applicant must be a resident of the U.S.A. I/We agree to comply with the safeguards printed on the reverse of this form, and understand that a permit may be subject to other conditions specified in Section B and C.* SIGNATURE OF APPLICANT *(Must be person named in Item 1)* | 18. DATE

**SECTION B - TO BE COMPLETED BY STATE OFFICIAL**

19. STATUS
☐ Approve ☐ Disapprove
☐ Accept USDA Decision

20. CONDITIONS RECOMMENDED

21. SIGNATURE | 22. TITLE | STATE | 23. DATE

**SECTION C - TO BE COMPLETED BY FEDERAL OFFICIAL**

## PERMIT

24. PERMIT NO.

***(Permit not valid unless signed by an authorized official of the Animal and Plant Health Inspection Service)***

Under authority of the Federal Plant Pest Act of May 23, 1957 or the Federal Noxious Weed Act of 1974, permission is hereby granted to the applicant named above to move the pests described, except as deleted, subject to the conditions stated on, or attached to this application. *(See standard conditions on reverse side).*

25. SIGNATURE OF PLANT PROTECTION AND QUARANTINE OFFICIAL | 26. DATE | 27. LABELS ISSUED | 28. VALID UNTIL | 29. PEST CATEGORY

PPQ FORM 526 (MAY 84) *Previous edition obsolete.*



BEST COPY AVAILABLE

**Carolina Biological Supply Company** will ship cultures based on your expected use date. When ordering, sufficient time should be allowed for proper handling. When no delivery date is given, shipment will be made at once. Orders are handled from the following locations:

Western USA, including Alaska & Hawaii
Powell Laboratories Division
19355 McLoughlin Boulevard
Gladstone, OR 97027
To order by phone call 1-800-547-1733, or FAX your order to 1-503-656-4208.

Eastern USA
2700 York Road
Burlington, NC 27215
To order by phone call 1-800-334-5551, or FAX your order to 1-919-584-3399. In North Carolina phone 1-800-632-1231.

The following microorganisms are available from Carolina Biological Supply:

| Microorganism | Catalogue No. |
|---|---|
| *Erwinia carotovora*** | 15-5045 |
| *Agrobacterium tumefaciens*** | 15-4825 |
| *Escherichia coli* (culture in tube) | 15-5065 |
| *Escherichia coli* (freeze-dried plus medium) | 15-5065A |
| *Escherichia coli* (culture on plate) | 15-5067 |
| *Escherichia coli* (culture in nutrient broth) | 15-5068 |
| *Fusarium oxysporum***, causes tomato wilt | 15-6033 |
| *Rhizoctonia solani*** | 15-6218 |

**Ward's Natural Science Establishment** suggests that delivery dates be specified for living cultures. Rush orders are available. Place orders by phone, 1-800-962-2660, Mon. - Fri., 8:00 am - 7:30 pm EST or by FAX, 1-800-635-8439, 24 hours. For international orders phone 1-716-359-2502 or FAX 1-716-334-6174. Their address is:

5100 West Henrietta Road
P.O. Box 92912
Rochester, NY 14692-9012

The following microorganisms are available from Ward's Natural Science Establishment:

| Microorganism | | Catalogue No. |
|---|---|---|
| *Erwinia carotovora*** | (freeze-dried with media) | 85-W-1858 |
| *Erwinia carotovora*** | (freeze-dried without media) | 85-W-1658 |
| *Agrobacterium tumefaciens*** | (living culture) | 85-W-0100 |
| *Agrobacterium tumefaciens*** | (freeze-dried with media) | 85-W-1810 |
| *Agrobacterium tumefaciens*** | (freeze-dried without media) | 85-W-1610 |

| Microorganism | Catalogue No. |
|---|---|
| *Agrobacterium tumefaciens* A208** (freeze-dried with media) | 85-W-1803 |
| *Agrobacterium tumefaciens* A208** (freeze-dried w/out media) | 85-W-1603 |
| *Agrobacterium tumefaciens* heat-curable** (living culture) | 85-W-0135 |
| *Escherichia coli* strain K-12 (freeze-dried with media) | 85-W-1864 |
| *Escherichia coli* strain K-12 (freeze-dried without media) | 85-W-1664 |

** Require a USDA PPQ 526 permit accompanying orders from some states. A copy is included in this chapter (see p. 24). These are plant pathogens and both Carolina Biological Supply and Ward's Natural Science Establishment restrict distribution in Alabama, Alaska, California, Florida, Georgia, Hawaii, Michigan, Puerto Rico, Tenessee and Wisconsin. Also, plant pathogens can be sent only to institutions, not to individuals.

**When a permit is required to obtain a pathogen, proper disposal of that pathogen is essential. (See the following section on disposal of pathogens and infected plants.)**

### *DISPOSAL OF PATHOGENS & INFECTED PLANTS*

The microorganisms used in these experiments are not dangerous to humans, however they may pose a risk to the plant life growing in your vicinity. Some are soilborne and should not be introduced into landfills. After completing each experiment, the refuse consisting of cultures, cheesecloth or swabs soaked with inoculum, inoculated plants and plant parts, should be sterilized prior to disposal (Figure 5.1).

Figure 5.1. *Plant materials and cultures in an autoclave bag and pan, ready for autoclaving.*

For agar cultures of pathogens, place culture plates in coffee cans, aluminum pans or in autoclave bags (available from scientific suppliers), and place tubes in a rack. Place these in an autoclave or pressure cooker and autoclave for 30 minutes at 15 psi pressure. Remove from the autoclave. Allow melted plastic plates to cool and agar to solidify, then discard. When cool enough to handle, pour media from glass tubes and plates into a beaker or other suitable container, allow the media to solidify, and then discard it. Wash and rinse glassware.

For broth cultures, cheesecloth, swabs, paper products soaked with inoculum, mortars and pestles, glassware, and utensils, either: 1) immerse in a solution of 10% bleach to soak for 24 hours or 2) autoclave for 30 minutes at 15 psi pressure. After 24 hours in 10% bleach or after autoclaving, discard liquid waste down the drain, throw solid waste in the garbage, and wash and rinse glassware.

For diseased plants and parts, place them in an autoclave bag or aluminum pan and autoclave for 30 minutes at 15 psi pressure. Remove from the autoclave, cool and discard. Soil should be sterilized in this manner also; but because the odor may be unpleasant to some people, it may be best to do this when school or class is not in session, over the weekend or in the late afternoon.

***IMPORTANT PUBLISHER'S NOTE: The microorganisms that were used in the development of experiments found in this publication (see Chapter 8) were obtained from ATCC, Carolina Biological Supply Company, and Ward's Natural Science Establishment, Inc. Information on ordering these microorganisms has been provided by the author for your convenience. This information does not constitute an endorsement by the National Association of Biology Teachers.***

# CHAPTER 6 Preparation of Sterile Media for Culturing Bacteria & Fungi

***Introduction***

Plant pathologists use many different types of media to culture the microorganisms that cause disease in plants. Most of their early work dealt with culturing fungi. The molds and mildews that cause plant diseases are grouped into two categories depending on whether they can or cannot grow on artificial media. Obligate parasites cannot be cultured; nonobligate parasites can be cultured. As researchers have become more sophisticated in developing culture media, fewer fungi have been categorized as obligate parasites. For instance, *Phytophthora infestans*, the fungus causing late blight of potato, was thought to be an obligate parasite until attempts to grow it on media containing dead flies were successful! While this example gives the reader an idea of the diversity in culture media for fungi, it fails to illustrate that most media consist of a base made from plant broth (e.g. lima bean agar).

When bacterial pathogens of plants were being discovered in the late 1800s, media for studying bacterial diseases of animals and humans were used. Today there are many types of culture media used just for plant pathogens. Current research in plant pathology on culture media concentrates on developing media that are selective for specific pathogens, thus eliminating the need for surface sterilization of plant parts, minimizing subculturing to obtain pure cultures, and facilitating isolation from soil. Four kinds of media are used in this monograph and are described below. They are simple to prepare and are routinely used to culture plant pathogens.

***The Process of Sterilization***

In college, university and private laboratories autoclaving is used to sterilize media and glassware. The autoclave accomplishes this by building up a pressure of 15 pounds per square inch (psi) and a temperature of about 275° F and maintaining these for a period of time, usually between 15 and 25 minutes. This publication will use 20 minutes as a general guideline.

Pressure cookers or table-top electric autoclaves are commonly used in high schools and other small laboratories. The pressure cooker is effective at sterilizing media and glassware provided it has been properly calibrated and maintained. Calibration of pressure cookers is sometimes done during the harvest and canning season by local Cooperative Extension offices. Again the recommendation is to maintain a temperature of 275°F and a pressure of 15 psi for 20 minutes.

Microwaving may be considered an effective way of sterilizing. However, due to the variability of microwaves and the risk that large batches of agar may explode or

Figure 6.1 (a) *Placement of glass petri plates in paper bag prior to autoclaving.*

Figure 6.1 (b) *Store unopened bag after autoclaving until ready to use.*

become superheated and boil over onto hands when being removed from the microwave, **this publication does not endorse the use of microwaves as a means of sterilizing media and glassware**.

*To sterilize dry glassware, utensils or cloth:*
Wrap or cover with aluminum foil. Place glass petri plates in clean coffee cans, small paper bags, or wrap in foil (Figures 6.1a and 6.1b). Autoclave for 20 minutes at 275°F and 15 psi. Fast pressure exhaust, or package as above and bake in oven at 350°F (180°C) for 2 hours.

*To sterilize media and liquids:*
Place in glass or autoclaveable plastic container of twice the volume (e.g. 500 ml in a 1-liter flask). Leave cap loose or seal with cotton plug covered with foil or with foil alone. Autoclave for 20 minutes at 275°F and 15 psi, then slow pressure exhaust.

Always wear protective gloves when removing media or glassware from the sterilizer to prevent burns. Be very careful when handling liquids that may boil over if jarred or swirled.

*To sterilize work surfaces:*
Spray surface with either 70% ethanol or 10% bleach. Wipe and allow to dry.

***Sterile Water Blanks***
Sterile water blanks are used for rinsing pieces of plant tissue after surface disinfecting; preparing dilution series; preparing inoculum; and providing moisture in plant incubations with pathogens. In biology they can have more applications than the ones in this publication.

Set up racks of culture tubes. With a large pipet, dispense 8-15 ml distilled water into tubes to fill up to 1/2 full. Some applications require a specific volume per tube. Cap with autoclaveable caps, screw caps or cotton. Loosen screw caps. Autoclave for 20 minutes, slow exhaust.

After autoclaving, tighten caps and allow to cool. Store in a clean, dry place or in the refrigerator.

***Media To Grow Bacteria***

NUTRIENT AGAR

This medium is a general bacteriological medium and is useful for growing bacteria, isolating single colonies, and storing bacterial isolates.

Sterile, premade plates and slants are available from Carolina Biological Supply Company and Ward's Natural Science Establishment.

Figure 6.2. *Flask of media on stir plate.*

**1. Preparation using dehydrated nutrient agar**

Measure out appropriate amount as indicated on container. Place in flask twice the size of volume being made. Add appropriate volume distilled water. Place a metal stirring rod into flask. On stir-hot plate, stir and heat until boiling and dissolved (Figure 6.2). Remove from stir-hot plate.

***For slants:*** Set up racks of culture tubes. With a large pipet dispense 8-10 ml into tubes to fill up to 1/3 full. Cap with autoclaveable caps, screw caps or cotton. Loosen screw caps. Autoclave for 20 minutes, slow exhaust.

After autoclaving, tighten caps and place slants at an angle of 30-40 degrees until agar solidifies. Store in a clean, dry place or in the refrigerator (Figure 6.3). 500 ml yields 50 to 60 slants (8 to 10 ml/slant).

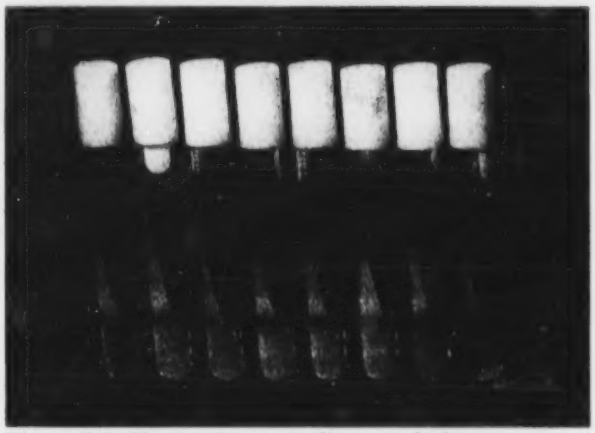


Figure 6.3. *Slants of agar medium ready for use.*

***For plates:*** Plastic plates are presterilized. Glass petri plates must be autoclaved. Stack in clean coffee

cans sealed with foil, wrap in foil, or place in small paper bags. Cap flask containing media with foil. Autoclave plates and media for 20 minutes, slow exhaust.

After autoclaving, place flask of media on stir plate and slowly stir until agar cools enough to handle. Agar will begin to solidify at 45° F. Surface sterilize work area by wiping down with either 70% ethanol or 10% bleach. Clean the surrounding area and keep air movement to a minimum. Pour media into sterile petri plates. Open the lid just enough to pour media into plate to cover bottom. Quickly replace the lid.

Allow media to solidify. To minimize condensation in the plates, stack them as they are poured. (Glass plates do not stack as well as plastic.) Leave plates at room temperature for 24 hours if condensation is severe. To prevent condensation from causing a wet agar surface, store plates upside down in clean plastic bags on a clean shelf or in the refrigerator. Prepare only what is needed for a given experiment because contamination may occur during prolonged storage.

500 ml yields about 20 plates (25 ml/plate). Pouring thinner plates will yield more plates per batch of agar, but with a shorter shelf life. For more than 20 plates, prepare batches of media in 500 ml volumes as described because media is difficult to pour from flasks larger than 1 liter.

**2. Preparation from scratch**

Ingredients

| | |
|---|---|
| Distilled water | 500.0 ml |
| Agar | 10.0 g |
| Beef extract | 1.5 g |
| Peptone | 2.5 g |
| Glucose | 5.0 g |

Place distilled water in a 1 liter flask; add agar. Place a metal stirring rod into flask. On stir-hot plate, stir and heat until boiling and agar dissolves. Remove from stir-hot plate. Add the rest of the ingredients. Place on stir plate and stir to dissolve. Proceed for either slants or plates as described above under nutrient agar.

NUTRIENT BROTH

Depending on the intended use of the broth, it may be sterilized in small flasks, bottles or tubes. The directions below use culture tubes.

Premade, sterile broth is available from Carolina Biological Supply Company and Ward's Natural Science Establishment.

**1. Preparation using dehydrated nutrient broth**

Measure out appropriate amount as indicated on container. Place in a flask twice the size of volume being made. Add appropriate volume of distilled water. Place a metal stirring rod into the flask. On stir plate, stir until dissolved. With a large pipet, dispense 10 ml/culture tube. Cap as for slants above. Autoclave for 20 minutes, slow exhaust.

After autoclaving, tighten caps and allow to cool. Store in a cool, dry place or in the refrigerator.

**2. Preparation from scratch**

Omit agar from agar recipe above. Dissolve ingredients by stirring. Dispense 10 ml/ culture tube. Cap loosely and autoclave for 20 minutes, slow exhaust.

After autoclaving, tighten caps and allow to cool. Store in a clean, dry place or in the refrigerator.

KING'S MEDIUM B

This medium is used to determine whether bacteria are fluorescent Pseudomonads. It is used in diagnostic laboratories and research laboratories for the identification of bacterial isolates.

If possible, prepare this medium from scratch because the dehydrated King's Medium B will not yield consistent results. If using dehydrated media, follow directions above for dehydrated nutrient agar plates.

Scientific supply companies may offer sterile, prepared plates of King's Medium B that may prove convenient and effective.

**Preparation from scratch**

Ingredients

| | |
|---|---|
| Distilled water | 500.0 ml |
| Proteose peptone #3 (Difco) | 10.0 g |
| $K_2HPO_4$ | 0.75 g |
| $MgSO_4 \cdot 7H_2O$ | 0.75 g |
| Agar | 7.5 g |
| Glycerol | 7.5 ml |

Place distilled water in a 1-liter flask; add agar. Place a metal stirring rod into flask. On stir-hot plate, stir and heat until boiling and agar dissolves. Remove from stir-hot plate. Add the rest of the ingredients except glycerol. Place on stir plate and stir to

dissolve. Add glycerol; stir to mix. Proceed for plates as described above under nutrient agar.

OVERLAY AGAR

See recipe under Water Agar in "Media To Grow Fungi" below.

***Media To Grow Fungi***

WATER AGAR

This medium is good for isolating fungi from soil or plant tissue pieces. The fungi derive most of their nutrition from the piece of plant tissue placed on the plate and will sometimes sporulate on this medium or on the plant tissue even though vegetative growth is sparse. By contrast, Oomycetes and Zygomycetes will grow out rapidly onto this medium, making it easy to obtain pure cultures of these microorganisms.

**Preparation**

Ingredients

| | |
|---|---|
| 2% water agar plates or slants | |
| Distilled water | 500 ml |
| Agar | 10 g |

Place distilled water in a 1-liter flask; add agar. Place a metal stirring rod into flask. On stir-hot plate, stir and heat until boiling and agar dissolves. Remove from stir-hot plate. Proceed for slants or plates as described under nutrient agar.

WATER AGAR AMENDED WITH STREPTOMYCIN

The Streptomycin antibiotic in this medium inhibits bacterial growth. This facilitates sporulation while maintaining purity.

Prepare stock solution of streptomycin sulfate:
In 200 ml (or greater) capacity brown medicine bottle, autoclave 100 ml water. Allow to cool to room temperature and add 1 g streptomycin sulfate. Cap and shake gently to dissolve thoroughly. This stock solution contains 10 mg/ml streptomycin (=10,000 ppm). (Instead of autoclaving, solution can be filter sterilized after streptomycin sulfate has dissolved.)

Figure 6.4. *Overlay agar medium in tubes.*

Prepare 500 ml batches of water agar as described above. When agar is cool and ready to pour, with a sterile pipet add 5 ml of the streptomycin stock solution to 500 ml of water agar to yield approximately 0.1 mg/ml (100 ppm) streptomycin. Swirl to mix the antibiotic thoroughly into the media.

Proceed for plates as described above under nutrient agar.

OVERLAY AGAR

Ingredients

| | |
|---|---|
| 1% water agar for overlays | |
| Distilled water | 500 ml |
| Agar | 5 g |

Proceed as above, but do not prepare plates. With a large pipet, dispense 7 ml/tube. After autoclaving do not slant. 500 ml yields 71 overlay tubes (See Figure 6.4).

POTATO DEXTROSE AGAR

This medium is a general mycological medium and is useful for growing fungi, transferring and maintaining pure colonies, and storing fungal isolates.

Sterile, premade plates and slants are available from Carolina Biological Supply Company and Ward's Natural Science Establishment.

**1. Preparation using dehydrated potato dextrose agar**

Measure out appropriate amount as indicated on container. Place in flask twice the size of volume being made. Add appropriate volume of distilled water. Place a metal stirring rod into flask. On stir-hot plate, stir and heat until boiling and dissolved. Remove from stir-hot plate.

Proceed for slants or plates as described under nutrient agar.

**2. Preparation from scratch**

Ingredients

| | |
|---|---|
| Distilled water | 500.0 ml |
| Potatoes, peeled and cut | 100.0 g |
| Agar | 10.0 g |
| Dextrose | 10.0 g |

Bring distilled water to boil, add potatoes, and cook until tender. Remove from heat and strain through cheesecloth-lined funnel to collect broth into a 1-liter flask. Pour into a 500-ml graduated cylinder and add distilled water to 500 ml. Pour back into

the flask and add agar. Place a metal stirring rod into the flask. On stir-hot plate, stir and heat until boiling and agar dissolves. Remove from stir-hot plate. Add the dextrose. Place on stir plate and stir to dissolve.

Proceed for either slants or plates as described above under nutrient agar.

ACIDIFIED POTATO DEXTROSE AGAR

The acid pH of this medium inhibits the growth of bacterial contaminants and can facilitate the isolation of pure cultures of fungi.

Prepare potato dextrose agar. After autoclaving, cool and then add 15-25 drops of 25% lactic acid /500 ml agar. Place on stir plate and mix.

Proceed for plates as described under nutrient agar. Individual plates can be acidified by dispensing one drop/plate and then pouring the agar over the drop and gently swirling to mix.

# CHAPTER 7 Preparation & Maintenance of Pure Cultures of Bacteria & Fungi

The maintenance of pure cultures requires knowledge of sterile technique. Sterile technique involves working in an environment and with methods that minimize contamination by microorganisms that are airborne or reside on surfaces. When working with fungi and bacteria, methods of sterile technique are similar. However, methods used to establish and transfer pure colonies of the organisms are different.

## BACTERIA

***Materials***

- Transfer or culture hood or a clean area for sterile transfer
- 10% bleach solution or 70% ethanol in a spray or squeeze bottle
- Paper wipes or cheesecloth
- Transfer loops
- Bunsen burners or alcohol lamps
- Matches
- Permanent marking pens or grease pencils
- Nutrient agar plates
- Parafilm®
- Nutrient agar slants
- Sterile water blanks

Figure 7.1. *Flaming the transfer loop.*

***Establishing Pure Cultures: Streaking To Obtain Isolated Single Colonies***

To sterilize work surfaces, spray surface with either 70% ethanol or 10% bleach. Wipe and allow to dry. When working without a transfer hood, minimize contamination by covering the work surface with a few layers of cheesecloth moistened in 10% bleach.

At the work area, place bunsen burner or alcohol lamp, transfer loops, marking pens, nutrient agar plates, parafilm, scissors, and plates from which transfers will be made.

Sterilize the transfer loop by flaming (Figure 7.1). Hold tip down over flame of alcohol lamp or bunsen burner until wire turns red hot. Also, flame the wire stem in this manner the first time. Cool loop on surface of the medium near edge of plate from which transfers will be made. When still somewhat hot the agar will sizzle and possibly splatter. Tiny drops of splattered agar can look like small bacterial colonies less than 24 hours old.

Figure 7.2. *Open plate only slightly as bacteria are streaked on the agar surface of plate.*

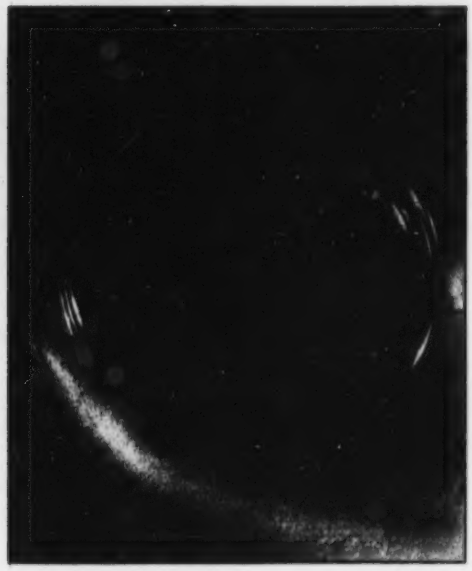


Figure 7.3. *Pick off a single, well-isolated bacterial colony by touching it with loop.*

Open plate lid only enough to work loop comfortably on agar surface. Keep lid over bottom. Work transfer loop with one hand and use other hand to hinge open plate lid (Figure 7.2).

Select bacterial colonies to be transfered. Hinge open plate lid just enough to pick off a single, well-isolated colony with sterile loop (Figure 7.3). Remove loop with bacterial cells and close lid. Hinge open a clean plate of nutrient agar. On one edge of circular agar surface, streak and then smear the bacterial cells on loop smoothly and evenly onto surface (Figure 7.3 and Figure 7.4a). Be careful not to gouge surface of agar with loop.

Flame loop to sterilize it and allow to cool. Rotate plate, positioning smeared area nearest you. Lift plate lid and place the loop onto the agar surface that was smeared. Run loop out onto untouched agar surface pulling bacterial cells onto this surface as shown in Figure 7.4b. Remove loop, replace lid, and flame loop. When loop is cool, rotate plate and repeat the streaking, only this time pull cells from second area out onto untouched agar (Figure 7.4c). Remove loop, cover plate, and flame loop. Rotate plate and repeat the streaking again from third area onto untouched agar (Figure 7.4d). Follow diagram in Figure 7.4. It is helpful, when first learning to streak bacteria for single colonies, to mark the bottom of the plate with a "K" as shown in Figure 7.5.

Transfer and streak at least one more bacterial colony of the same type onto another plate. Label plates with the name of bacterial isolates and date of transfer. Wrap

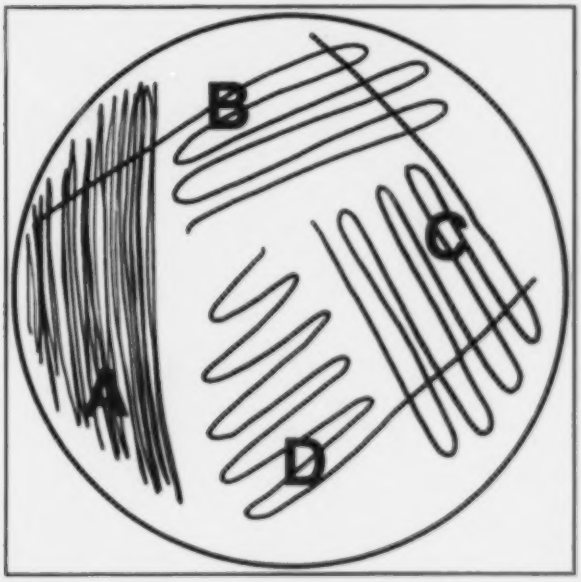



Figure 7.4. *a) After picking up a well-isolated colony with loop, thoroughly and evenly spread the bacteria on the loop over this area of the agar surface. b) With flamed and cooled loop, pull bacteria from area (A) over fresh area in a streak as shown. c) With flamed and cooled loop, pull bacteria from area (B) over fresh area in a streak as shown. d) With flamed and cooled loop, pull bacteria from area (C) over fresh area in a streak as shown.*

parafilm around edge of plate to hold lid on and prevent contamination by culture mites. Incubate streaked plates upside down at room temperature.

In 24 to 48 hours look for single colonies of bacteria in the second, third or fourth areas (Figure 7.4b, 7.4c, 7.4d).

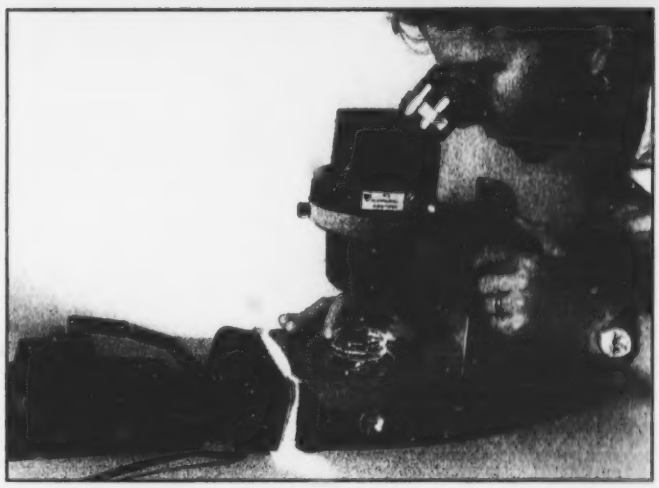
Check colonies for purity by looking at them under good light and by observing them under a dissecting microscope, preferably with transmitted light (light that shines up through the colonies (see Figure 7.6). The plates are pure if there is only one type of bacterial colony. Cultures derived from ATCC, Carolina Biological Supply Company and Wards Natural Science Establishment, Inc. orders should be pure. If not, contact the supplier as soon as possible. Cultures that have been subcultured and stored run the risk of becoming contaminated. Keep a description of the colony morphology of the different bacteria being stored for future reference. When streaking to obtain single colonies it may be possible to pick out the desired bacterial colony from other types of bacteria on the plate.

***Growing Isolates for Experimentation***

Once a pure culture is obtained, transfer single colonies to plates using sterile technique by picking up an individual colony with a sterile loop and streaking this across the entire surface of a clean plate. The cultures will be ready for experimentation in 24 to 48 hours when grown at room temperature.

***Storage***

Bacterial isolates may be stored rather than ordered from the supplier each year,



Top left: Figure 7.5. *Mark the bottom of the plate with a "K" and use the spaces formed by the lines of the "K" as A, B, C and D described in Figure 7.4.* Bottom: Figure 7.6. *Dissecting microscope with transmitted light.* Top right: Figure 7.7. *Flaming the end of a tube prior to transfer of bacteria.*

provided the biology program has time and space to store cultures. Isolates are best stored in a refrigerator as a suspension of cells in sterile water blanks, but may be stored on nutrient agar slants (see Chapter 7). They can also be stored on plates, but these require more storage space and run greater risk of contamination during maintenance transfers. Bacteria in the genera *Erwinia* and *Agrobacterium* are best stored in sterile water to prevent the loss of viability.

*Storage in Sterile Water Blanks*

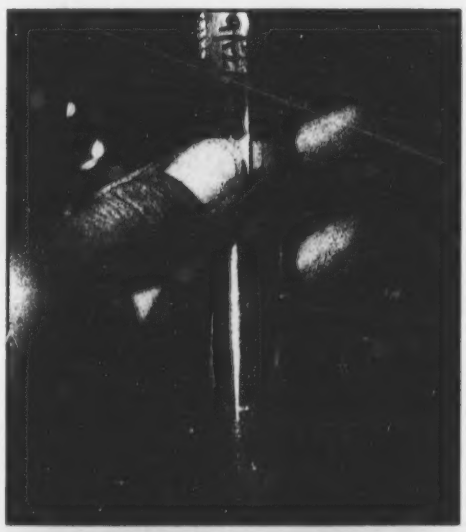
Remove cap and pass end of water blank tube over flame. Hold the cap between two fingers of the hand holding the tube. This avoids contaminaton from setting it down. Flame loop and cool as described above and transfer desired bacterial colony into sterile water. Reflame end of tube and replace cap. Vortex to mix thoroughly. Label

and date cultures, include date of original isolation. Place sterile water culture in the refrigerator soon after transfer. For storage in water, use screw cap tubes or tape the cap on tightly to prevent evaporation.

*Storage on Nutrient Agar Slants*

Remove cap and pass end of tube over flame (Figure 7.7). Flame loop and cool as described above, then transfer desired bacterial colony to surface of agar slant (Figure 7.8). Reflame end of tube and replace cap. Incubate slant at room temperature and check for growth after 24 hours. If growth has occurred, store slant in refrigerator. Label and date slants, including date of original isolation.

Periodic transfers of isolates in storage are necessary to make certain they are viable. Once per month or at most every 6 months, streak out each isolate onto a plate. After 24 hours at room temperature, check for growth, colony characteristics, and purity. If possible, check for virulence (i.e. ability to cause soft rot, crown gall, etc.). If isolate is viable, place it in storage again.



Top: Figure 7.8. *Transfer of bacteria to surface of slant.* Bottom: Figure 7.9. *Flaming a dissecting tool. Tip is bent at 90° angle to facilitate transfer of agar cube.*

## FUNGI

***Materials***

- Transfer or culture hood or a clean area for sterile transfer
- 10% bleach solution or 70% ethanol in a spray or squeeze bottle
- Paper wipes or cheesecloth
- Dissecting needles
- Bunsen burners or alcohol lamps
- Matches
- Permanent marking pens or grease pencils
- Potato dextrose agar plates
- Parafilm®
- Potato dextrose agar slants

***Establishing Pure Cultures***

When working with fungal cultures use the

Top: Figure 7.10. *Cutting a small cube of agar from the fungal colony's edge.* Middle: Figure 7.11. *Transfer of cube of agar containing bits of fungal hyphae to center of fresh plate.* Bottom: Figure 7.12. *Transfer of fungal colony to slant.*

sterile techniques described under bacteria: sterilize surfaces with 10% bleach or 70% ethanol, flame tools (Figure 7.9), open plates only slightly.

To transfer fungal colonies: using a flamed dissecting needle cut a small (3-5 $mm^3$) cube of agar containing bits of fungal hyphae from the colony's edge (Figure 7.10). Pick up the cube by stabbing it with the dissecting needle and transfer it to the center of a clean plate (Figure 7.11). Repeat at least once with each isolate. Wrap Parafilm around edge of plate to hold lid on and prevent contamination by culture mites. Label and date plates. Incubate transfers at room temperature. Observe daily for fungal growth. Invert plates if condensation becomes a problem.

After plate is almost covered by mycelium, sporulation of fungal cultures can be stimulated by light/dark cycles. Cultures respond well to growth under shortwave UV light on a 12-hour daylength.

Keep a description of the colony morphology of each fungus for reference. Fungal mycelia grow out on agar from a central point in a circular pattern. Their appearance might be fluffy, or flat, or red, or white, or slimy, etc. Pure cultures are uniform in appearance, although *Fusarium* species may have a tendency to sector. Sectoring is when wedges of slightly different morphologies occur in the circular colony.

*Storage*

Fungi are usually stored on slants of potato dextrose agar, but can be stored on plates. Cut a small (3-5 $mm^3$) cube of agar containing bits of fungal hyphae from colony's edge. Pick up cube by stabbing it with dissecting needle. Transfer cube to center of plate. For slants: Remove cap from slant and pass end of tube over flame. Transfer cube to slant, reflame tube end, and replace cap (Figure 7.12). Label and date the slant, including date original isolate was made. Incubate at room temperature and observe daily for growth. When substantial growth has occurred and transfer appears pure, refrigerate for storage. Make monthly (maximum 6 months) transfers to maintain viability of isolates. Wrap edges of plates with Parafilm or tape to seal the lid and reduce dehydration of agar.

**NOTE: Pathogens kept in storage for long periods of time have been known to lose viability or virulence. For this reason it may be necessary to reorder isolates from suppliers after several years of storage.**

# EXPERIMENTS

## A. The Symptoms & Signs of Disease

*Introduction*

The symptom of a disease is the appearance or manifestation of changes in a plant resulting from its reaction to a pathogen or injurious agent. The signs of a disease are the appearance of the pathogen itself. To study plant diseases both the symptoms and the signs of the disease must be characterized. This can be very challenging because the signs of disease are very small, microscopic (e.g. fungi) to submicroscopic (e.g. viruses), and can be easily overlooked; plant symptoms may be subtle and difficult to distinguish from normal, healthy tissues.

To impart a working knowledge of symptoms and signs on plants, a glossary of common terms is given below.

**SYMPTOMS**

**Blast**: The failure of a bud to develop and bloom, especially a flower bud.

**Blight**: The rapid yellowing, browning and death of flowers, leaves, shoots, stems, or the whole plant (Figure 8A.1).

**Canker**: A dead area on a stem. A canker may grow all the way around a stem and girdle it, killing the parts above it (Figure 8A.2).

**Chlorosis**: Yellowing of normally green plant parts. Chlorosis occurs when chlorophyll is not made by the plant or is destroyed by the pathogen (Figure 8A.3).

**Color break**: The change to another color of irregular areas, usually on flower petals but also on vegetable fruits (Figure 8A.4).

**Curl**: The puffing out of portions of a leaf that causes the whole leaf or parts of it to blister or cup (Figure 8A.5).

**Damping off**: The death and collapse of the stem of a seedling at ground level, causing it to fall over (Figure 8A.6).

**Decay**: The breakdown or decomposition of plant tissue, especially the woody tissue of plant stems and roots.

**Dieback**: The death of the tips of stems or shoots or the failure of branches to grow leaves (Figure 8A.7).

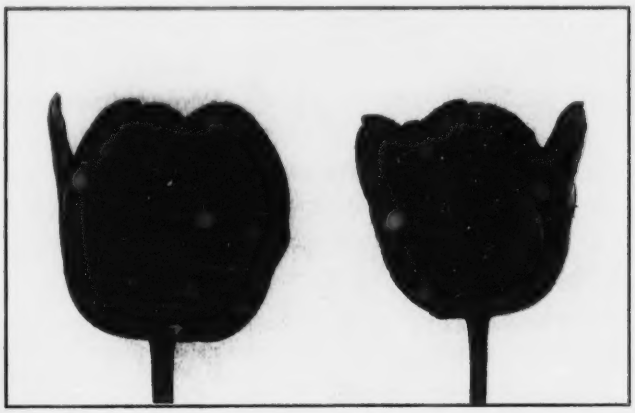
**Fasciation**: The growing closely together or fusion of many buds, shoots, or stems where only one should grow (Figure 8A.8).

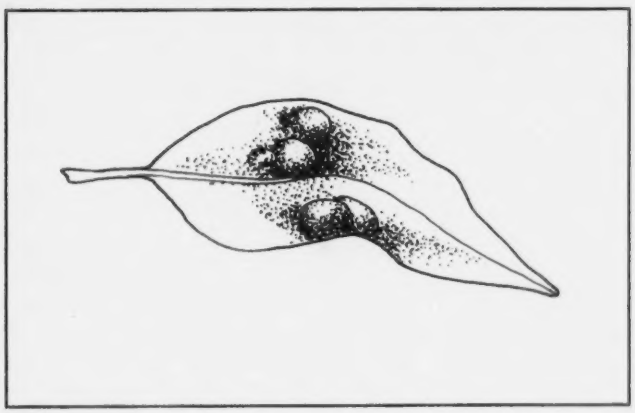
**Gall**: A large swelling or abnormal outgrowth of a plant part (Figure 8A.9).

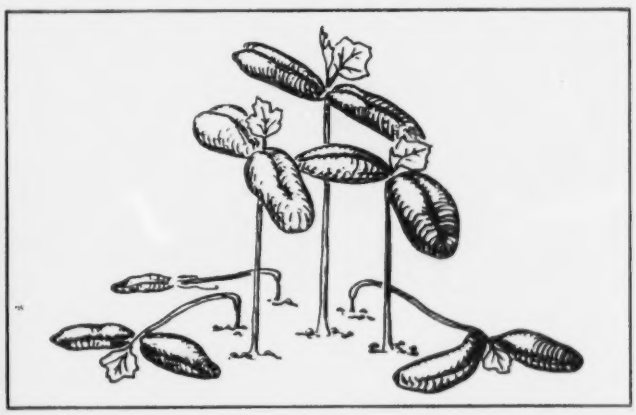
**Lesion**: A localized area of diseased or dead tissue found on roots, stems, fruits and leaves.

**Mosaic**: A blocky, angular pattern of yellowing, light green, and dark green colors on leaves (Figure 8A.10).

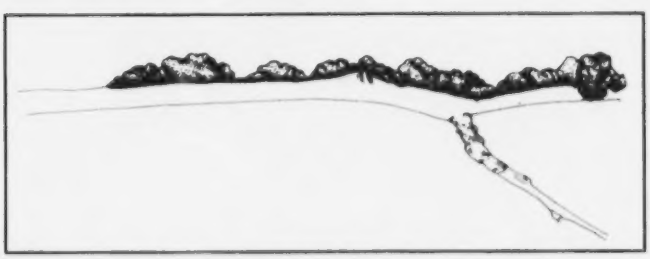
**Mummy**: The shriveled remains of a fruit or vegetable still attached to the plant or dropped to the ground (Figure 8A.11).







Top left: Figure 8A.1. *Botrytis blight of geranium caused by the fungus* Botrytis cinerea. Middle left: Figure 8A.2. *Canker on cherry stem caused by the fungus* Nectria galligena. Bottom left: Figure 8A.3. *Chlorosis of rhododendron leaves induced by a micronutrient deficiency.* Top right: Figure 8A.4. *Color break on tulip caused by tulip breaking virus. Healthy tulip on left.* Middle right: Figure 8A.5. *Peach leaf curl caused by the fungus* Taphrina deformans. Bottom right: Figure 8A.6. *Damping off of cucumber seedlings caused by one of several fungi.*



Top: Figure 8A.7. *Dieback of branches, a common symptom of maple decline.* Middle: Figure 8A.8. *Fasciation of snapdragon stems, cause unknown.* Bottom: Figure 8A.9. *Galls of the black knot disease of plum and cherry caused by the fungus* Apiosporina morbosa.

**Necrosis**: Browning or blackening of areas on a plant.

**Ringspot**: A yellow ring-shaped area with a green center occurring on leaves (Figure 8A.12).

**Rosette**: The crowding of leaves caused by the failure of shoots to grow in length.

**Rot**: The wasting away or breakdown of plant parts, especially fruits and vegetables, roots, crowns, seeds, and other storage organs.

**Russet**: Rough and light brown areas on the surface of fruits and vegetables (Figure 8A.13).

**Scab**: A crustlike brown to black spot on a plant leaf, fruit, or storage organ (Figure 8A.14).

**Scorch**: The drying out and browning of the tips and edges of leaves (Figure 8A.15).

**Shot hole**: The dropping out of many small, circular, necrotic spots from leaves.

**Soft rot**: The breakdown of plant parts into a gooey, liquid mass, often having a bad odor.

**Speck**: A small, brown, dead spot less than 1/16 inch or 2 mm across. Specks often occur in groups of many (Figure 8A.16).

**Spot**: A dead or injured area on a leaf or fruit.

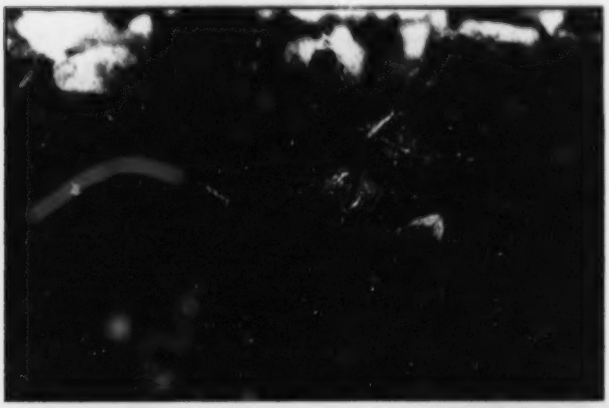
**Stunt**: The failure of plants or their parts to grow to a normal size.

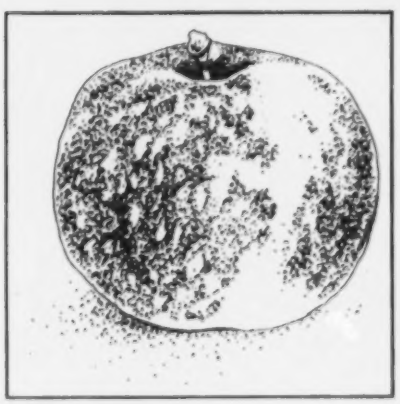
**Wilt**: The sagging or drooping of leaves, young shoots, or the whole plant from lack of water traveling through the vascular system of the plant or plant part.

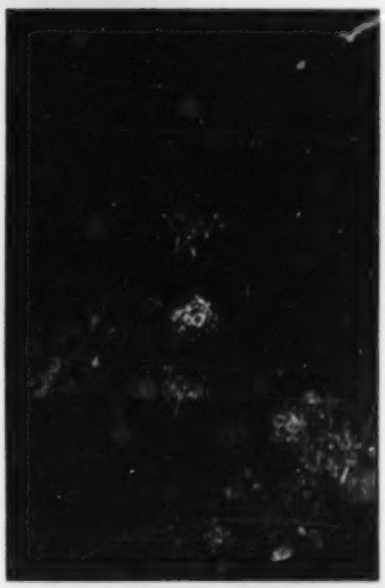
**Witches' broom**: The growth of many shoots and stems from the same point on the plant giving a bushy or broomlike appearance (Figure 8A.17).

**Yellows**: The entire plant is yellow instead of green.

The above symptoms are rarely found separately on a diseased plant. For instance, many leaf spots on one leaf may expand rapidly and blight the leaf. Fas-





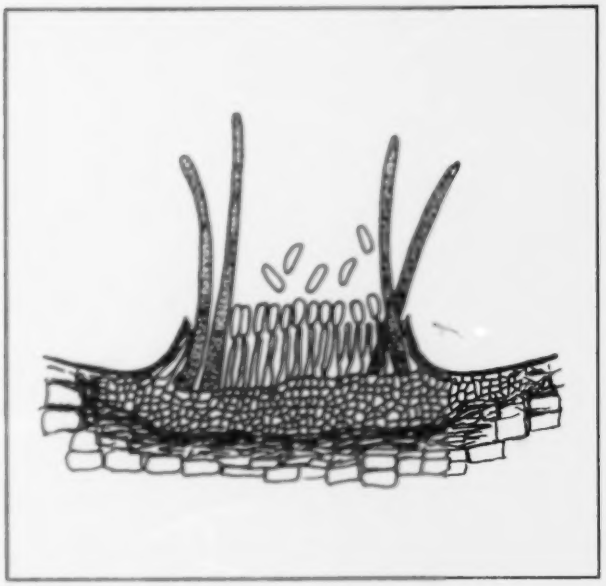


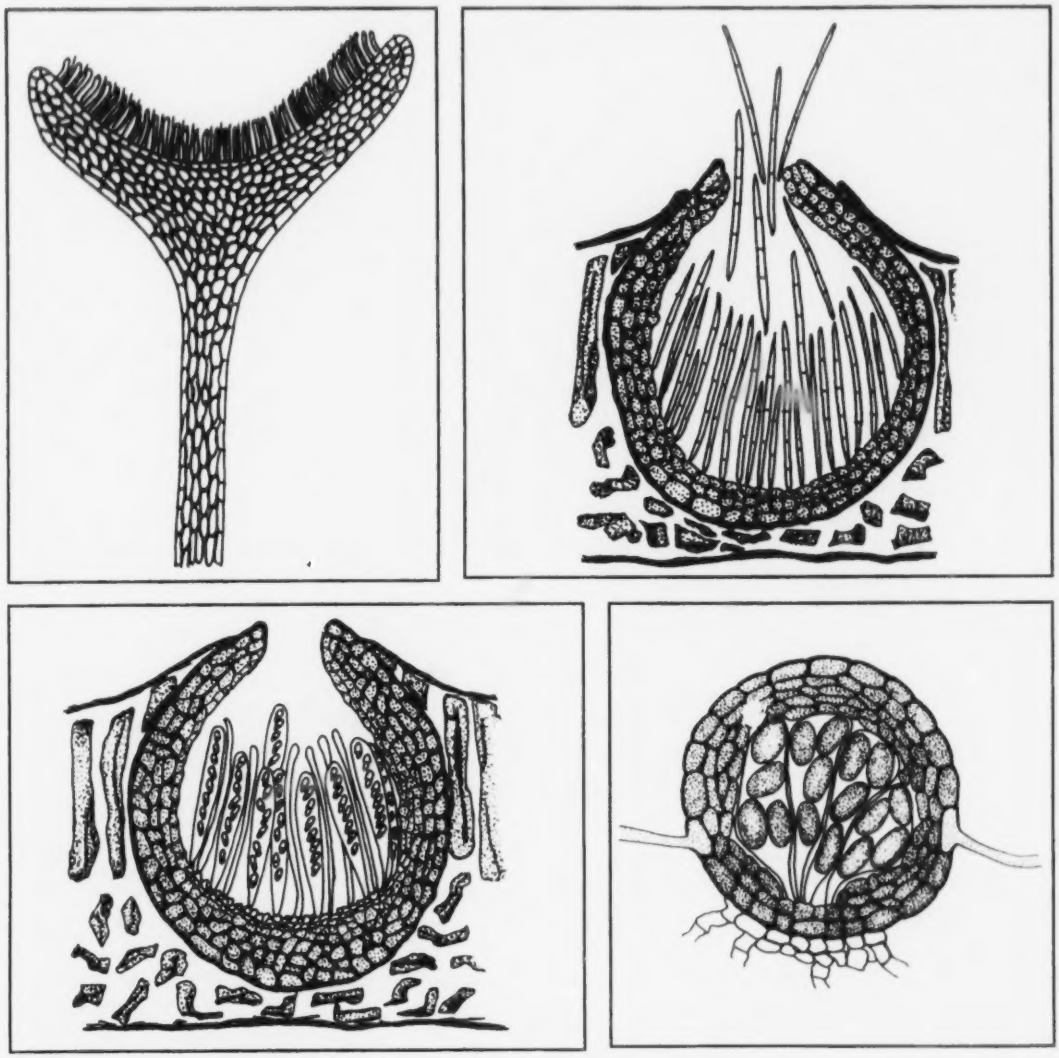
Top: Figure 8A.10. *Mosaic on bean leaves caused by bean common mosaic virus.* Middle left: Figure 8A.11. *Shriveled fruit mummies and rotting berries on a grape cluster associated with the disease black rot caused by the fungus* Guignardia bidwellii. *Note the tiny dots on the rotting berries. These are the fruiting bodies or pycnidia of the* Phyllosticta *stage of the fungus.* Middle right: Figure 8A.12. *Ring-spot and line patterns on leaves and fruit of bell pepper caused by cucumber mosaic virus.* Bottom left: Figure 8A.13. *Russet on apple skin may be caused by powdery mildew and a variety of other factors.* Bottom right: Figure 8A.14. *Common scab of potato caused by the bacterium* Streptomyces scabies.

ciated stems are often found in witches' brooms. Bud blast can signal the possibility of shoot blight or dieback injury. Root rot is often associated with stunting and wilting of the plant. Dieback of a shoot may lead to a canker on the stem to which the shoot is attached. Or a canker on a large stem may cause dieback of the shoots growing from it.

Top: Figure 8A.15. *Leaf scorch on maple caused by deicing salt applied to roadway near tree* Middle left: Figure 8A.16. *Fly speck of apple caused by the fungus* Microthyriella rubi. Middle right: Figure 8A.17. *Witches' brooms on willow branches, cause unknown.* Bottom left: Figure 8A.18. *a) Cross-section view of an acervulus.* Bottom right: Figure 8A.18. *b) Apothecia growing out of a mummified fruit.*

OPPOSITE PAGE: Top left: Figure 8A.18. *c) Cross-section view of a single apothecium.* Top right: Figure 8A.18. *d) Cross-section view of a pycnidium.* Bottom left: Figure 8A.18. *e) Cross-section view of a perithecium.* Bottom right: Figure 8A.18. *f) Cross-section view of a cleistothecium.*





## SIGNS

**Fruiting body**: The structure in or on which a fungus produces its spores. These may be visible as tiny dots no larger than the period at the end of this sentence. Some of the fruiting bodies commonly found on diseased plant tissues include (Figure 8A.18):

A. Acervulus (pl. _i) - an open, cup-like structure containing conidia.

B. Apothecium (pl. _a) - an open, cup-like structure containing ascospores.

C. Pycnidium (pl. _a) - a round structure with an opening at the top containing conidia.

D. Perithecium (pl. _a) - a round structure with an opening at the top containing ascospores.

E. Cleistothecium (pl. _a) - a spherical structure without an opening containing ascospores.

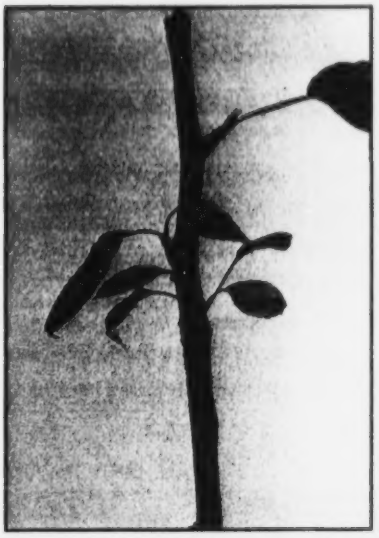


Figure 8A.19. *Bacterial ooze issuing from an apple branch infected with fireblight caused by the bacterium* Erwinia amylovora.

**Mildew**: The white powdery or downy growth of a fungus on leaves, shoots, flowers or fruits.

**Mold**: The fuzzy growth of a fungus on a plant part.

**Mycelium**: The threadlike, weblike, or matlike growth of a fungus.

**Ooze**: The amber or cream-colored drops of liquid containing bacteria that drip or seep from infected plant parts (Figure 8A.19).

**Rust**: The red, yellow, orange, or sometimes black fruiting bodies or spore masses seen on plants infected with rust fungi.

**Sclerotium** (pl. _a): A hard, durable structure produced by certain fungi that allows the fungus to survive in soil or plant debris for many years. Sclerotia are about the size of a BB and have a tough brown to black rind and a uniform, white interior.

**Spore**: Spores are the reproductive structures of fungi. Fungal spores may be seen when many are grouped together on the surface of mildew, mold or mycelium, or when they ooze out of a fruiting body in droplets or tendrils. Conidia are vegetatively or asexually produced spores. Ascospores are sexually produced spores, contained in a sac called an ascus.

***Purpose***

•To collect plants that have symptoms of disease.
•To study diseased plants and describe the symptoms and signs of disease.
•To learn how to look for microscopic signs of disease.

***Materials***

Various plant specimens showing symptoms collected by the class. Include mildews, rusts, leaf spots, and fruit rots. Pine cones, dead treé branches, drift wood, and compost heaps are good places to find fungal fruiting structures, although these might not be of pathogenic fungi. When collecting plant specimens on field trips always be aware of the poisonous or rash-producing plants in your area and take precautions to avoid them.

- •Hand lenses, magnifying glasses.
- •Baskets and bags.
- •Trowel, sturdy field knife, pocket knife, pruning shears.
- •Notepad or index cards and pencils.
- •Dissecting and compound microscopes.
- •Microscope slides and cover slips.

•Dissecting needles and knives and single edge razor blades.
•Moist chambers in which to incubate specimens (petri dishes, glass or plastic containers with lids, or plastic bags lined with wet towels or filter paper).
•Acidified Potato Dextrose Agar (APDA) or Water Agar (WA) or WA amended with streptomycin or Nutrient Agar (NA) plates (see Chapter 6).
•Surface disinfectant (10% bleach or 70% ethanol).

***Methods***

Read the safety information in Chapter 4 on bleach and ethanol. All other chemicals pose little hazard, but always read the chemical label on the container.

Plan a field trip or series of field trips to collect diseased specimens. Vegetable gardens, flower gardens, corn fields, vegetable fields, abandoned orchards, and your own yard or city's park or botanic garden should provide ample material. Try to collect a few specimens with a few obvious symptoms and, if possible, avoid those symptoms that may be insect damage or nutrient deficiencies. Also, collect healthy plant specimens for comparison.

On the field trips take baskets and bags for collecting, hand lenses or magnifying glasses for close examination of plants, tools to trim, dig, or cut samples, and notepad and pencils for notetaking. A field guide to plants may prove helpful (Figure 8A.20).

As specimens are collected, write down the plant's name, the location collected from (town, state, county), and the collector's name on a label and keep the label with the specimen. Back in the classroom, spread the collected specimens out on the lab bench. Write on the blackboard a list of symptoms and their definitions.

Have each student or group of students choose a plant specimen to work with: Describe the symptoms on each plant specimen. Look closely at the plant specimens for evidence of signs of a pathogen (most will be microscopic in size). Begin examinations with a hand lens and dissecting microscope. Write down the observations of signs.

If mildew, mold or mycelium is seen, scrape it with a razor blade or scalpel into a drop of water on a slide. Stir scrapings into the water, place a coverslip over them and observe with compound microscope. If fruiting bodies are found on diseased portions, use dissecting needles, razor blades, scalpels or knives to tease them off and place into a drop of water on a slide. Place a coverslip on top and squash with eraser end of pencil. Alternatively, a squarish chunk of tissue may be removed from area of interest on specimen and ultra-thin cross sections through fruiting bodies may be made with a razor blade. Place sections in water on a slide, cover with coverslip, and examine with compound microscope. Depression slides may facilitate viewing of larger sections and scrapings.

To favor growth of fungi from diseased specimens and to soften them to facilitate dissection, place them in moist chambers and observe daily for appearance of fungal

Figure 8A.20. *Field trip supplies for collecting plants with symptoms and signs of disease.*

fruiting bodies, mycelial growth, or bacterial ooze. Tease fungi off specimen and mount in water on a slide. Observe with compound microscope.

If desired, cultures may be made from the diseased tissue onto APDA, WA or WA streptomycin plates to recover fungi or NA plates to recover bacteria. Surface sterilize the specimen: Rinse in water and soak in 10% bleach solution for 2-5 minutes. Remove and let air dry on a clean paper towel. Using sterile technique (Chapter 6), excise tiny bits of tissue from the margin between diseased and healthy tissue making sure to include some of both, but mostly healthy tissue. Place these bits onto agar plates and watch for growth of fungi or bacteria. Subculture from advancing hyphal tips or bacterial colonies (see Chapter 7).

From fruits such as pumpkins or apples that have distinct rotten areas, cultures may be quite successful and the cultured microorganisms may be inoculated back into healthy fruits in an attempt to recreate the symptoms using Koch's Postulates (see Experiment B).

***Results***

A variety of plant symptoms should be observed. Some signs of disease also should be found. The primary purpose is not to identify the diseases, but rather to observe the symptoms and signs and to learn the difference between these two terms. The fungi found in the moist chambers, in culture, or on the plant surfaces are not necessarily plant pathogens. They may be merely surface inhabitants or saprophytes growing on tissue killed by something else. This is why Koch's Postulates play an important role in plant pathology.

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## B. Koch's Postulates

***Introduction***

Koch's postulates of disease causation were devised to verify the cause of an infectious disease since simple association of an organism with a disease is insufficient evidence that it is the causal agent. They are known as the Rules of Experimental Proof. In plant pathology they are used as follows:

a. The pathogen must be found associated with the disease in all the plants examined.

b. For nonobligate parasites, the pathogen must be isolated, grown and characterized on nutrient media. For obligate parasites, the pathogen must be isolated and grown on a susceptible host plant and its appearance and effects on the plant recorded.

c. The pathogen from pure culture must be inoculated onto healthy plants of the same species or cultivar on which the disease occurs and it must produce the same disease on the inoculated plants.

d. The pathogen must be isolated in pure culture from the inoculated plants and its characteristics must be exactly like those observed in Step b.

The disease Fusarium wilt of tomato is caused by the soilborne fungus *Fusarium oxysporum* forma specialis *lycopersici*. This fungus invades through the roots of the tomato plants. Its spores (microconidia) and hyphae colonize the xylem of the plant, plugging the water-conducting vessels. Water, inefficiently transported through the plugged vessels, and toxins produced by the fungus, cause the infected plant to become wilted and yellowed, respectively. The disease manifests intself in the stunted, yellowed and wilting plant that may eventually die (Figure 8B.1).

***Purpose***

•To study the fungus *Fusarium oxysporum* f. sp. *lycopersici*, its colony morphology, hyphae and spores.
•To inoculate *Fusarium* into tomato and observe symptoms.
•To isolate *Fusarium* from tomato.
•To compare the isolated fungus with the one used as inoculum.

***Materials***

•Tomato plants, cultivar New Yorker, or a cultivar that is NOT RESISTANT to Fusarium wilt. Resistance is usually denoted by the letter F on the seed packet, seed catalog, or plant label.
•Sterile potting mix.
•4" or 6" pots, washed and sterilized. To sterilize - Autoclave clay pots (60 minutes, fast exhaust); soak clay or plastic pots in 10% bleach for 1/2 to 2 hours and rinse thoroughly.

•Cultures of *Fusarium oxysporum* f. sp. *lycopersici* growing on acidified potato dextrose agar (APDA) plates (Figure 8B.2a) (see Chapters 5 and 7).
•Water agar (WA) amended with streptomycin (see chapter 6).
•Acidified potato dextrose agar plates (see Chapter 6).
•Potato dextrose agar (PDA) slants (see Chapter 6).
•Dissecting knives.
•Compound microscope.
•Dissecting microscope.
•Microscope slides and cover slips.
•Sterile water blanks (see Chapter 6).
•Sterile rubber policemen or bent glass rods.
•Surface disinfectant (10% bleach or 70% ethanol).
•Two 250- or 500-ml beakers.

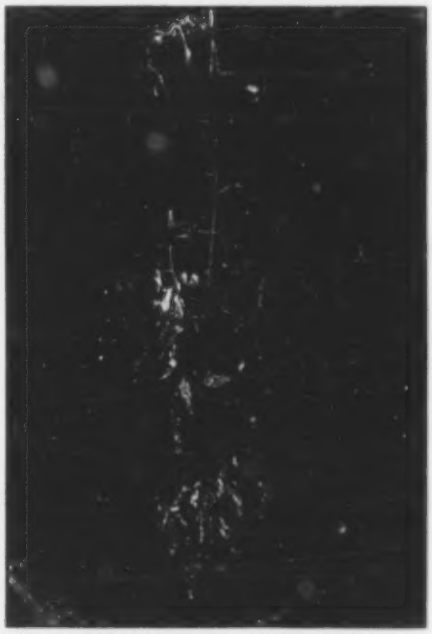


Figure 8B.1 *Tomato plant affected by Fusarium wilt shows severe wilting and yellowing.*

***Methods***

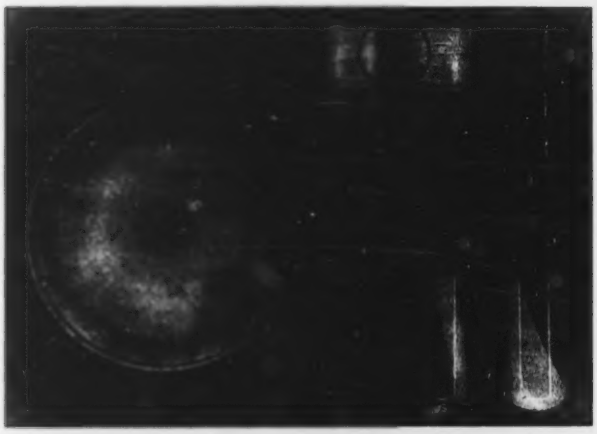
Read safety information in Chapter 4 on bleach and ethanol. All other chemicals pose little hazard, but always read the chemical label on the container.

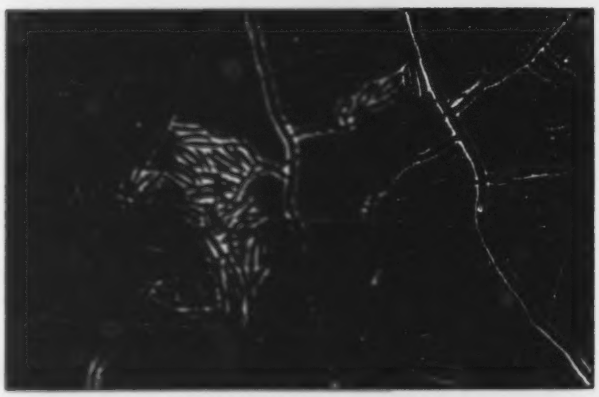
Grow tomato plants for 4 weeks or until 3-5 inches tall. Use clean, surface disinfected pots and sterile potting mix. Tomato bedding plants may be purchased at this stage just prior to the local gardening season. Grow nonresistant varieties.

Prepare plates and slants listed under materials (see Chapter 6).

Transfer pure cultures of *F. oxysporum* f. sp. *lycopersici* to 10 or more WA streptomycin plates and two PDA slants (see Chapter 7). Place transfers under fluorescent lights with a 12-hour light and 12-hour dark cycle to stimulate sporulation on WA and enhance development of characteristic colony morphology on PDA. Allow fungus to grow for 10-14 days.

Examine the fungus:
Describe colony appearance growing on PDA slants (Figure 8B.2b). Is colony sparse or dense, white or pigmented, flat or fluffy? What color is it? Color is best seen on PDA slants. (Flat and slimy colonies have mutated into "pionnotal" forms and will no longer sporulate.) Store slants in refrigerator.





Top: Figure 8B.2. *a) 14-day-old culture of* Fusarium oxysporum *f. sp.* lycopersici *growing on acidified potato dextrose agar. b) Two potato dextrose agar slants of* Fusarium oxysporum *f. sp.* lycopersici *showing pigmentation, next to a clean slant.* Middle: Figure 8B.3. *a) Hyphae and macroconidia of* Fusarium oxysporum *f. sp.* lycopersici *as seen through a compound microscope.* Bottom: Figure 8B.3. *b) Macroconidia and microconidia of* Fusarium oxysporum *f. sp.* lycopersici *as seen through a compound microscope.*

Using sterile technique (Chapter 6) and one of the WA cultures, place bits of fungal mycelium (the mat of hyphae growing on agar surface) into small drop of water on slide. With dissecting needles, tease apart to spread mycelium out in drop of water, freeing up the individual hyphae (Figure 8B.3a). Cover with coverslip and examine under microscope. Look for spores of the fungus (Figure 8B.3) and describe and illustrate them. *Fusarium* has two types of spores (conidia): macroconidia and microconidia. Macroconidia are the "big banana" ones with cross walls. Microconidia are the small, non-descript ones. *Fusarium oxysporum* also produces chlamydospores, round, thick-walled resting spores. Note which types of spores are present. Spores and hyphae of *Fusarium* are hyaline (colorless) when viewed through a microscope.

If spores are not found, grow colonies for two more days and recheck the same plate checked before. Allow other plates to grow undisturbed. When spores (conidia) are observed, flood each of the undisturbed plates with 10 ml sterile water: Pour water onto surface of fungus growing on plate. Harvest spores by rubbing surface of colony with either a sterile rubber policeman or a sterile bent glass rod. Pour spore suspension into a 250-ml or a 500-ml beaker.

Remove tomato plants from soil in which they are growing and wash roots in running water to remove most of potting mix.

Dip and swirl root system of tomato plants into spore suspension in beaker. Dip and swirl roots of at least two tomato plants in sterile water only. These will serve as controls. Immediately replant inoculated plants, one per pot, in fresh potting mix.

Grow tomatoes in a sunny location. Observe plants 2-3 times per week for development of symptoms. This should take 2 to 3 weeks.

Before symptoms develop, prepare APDA and WA streptomycin plates, if necessary. These will be used to reisolate the fungus from inoculated plants.

When symptoms develop, remove symptomatic and control plants from soil and wash roots in running water. Compare healthy and diseased plants and describe symptoms.

Carefully dissect one healthy and one diseased plant. Cut lengthwise just below epidermis at the base of stem to expose xylem. Note differences between healthy and diseased xylem tissues.

Select three or more intact, diseased tomato plants from which to reisolate. Also isolate from an intact healthy tomato plant.

*Reisolate from plants:*
Trim off leaves and secondary roots leaving only main stem, hypocotyl and main root. Mark or tag healthy stem. Soak stems in 10% bleach solution for 5 minutes. Remove pieces and place on paper towels to dry. Using sterile technique, cut thin (2 to 4 mm thick) wedges out of one side of stem near root/stem junction (Figure 8B.4). Include xylem tissue with each wedge. Cut several wedges and, with sterile forceps and using sterile technique, place wedges onto an APDA and a WA streptomycin plate. Place 5-6 wedges on each plate, one in the middle and others spaced evenly around center piece. Incubate plates under fluorescent lights. With a dissecting

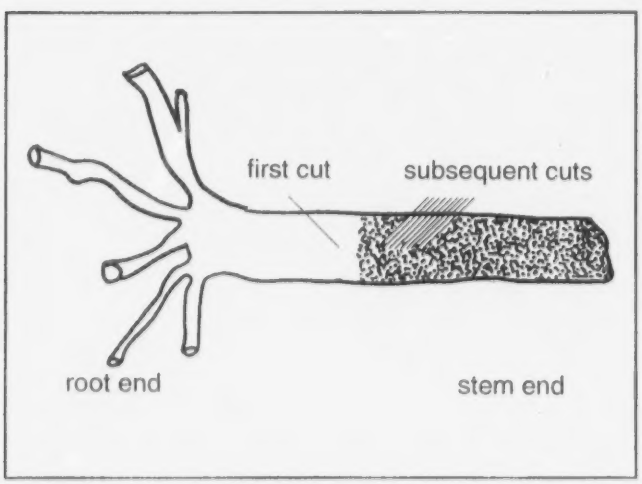



Figure 8B.4. *Diagram of isolation of vascular wilt pathogens from xylem. Make first cut at an angle. Make subsequent cuts at an opposing angle to remove thin xylem sections.*

microscope, check plates every 2 days for fungal hyphae growing from xylem.

From APDA, transfer tips of fungal hyphae to clean APDA and WA streptomycin plates (see Chapter 8). Under fluorescent lights, grow transfers and check for characteristic growth. Examine WA streptomycin plates for sporulation on the xylem-containing tissue piece. Transfer *Fusarium* colonies to APDA and WA streptomycin plates and grow out, as above. Examine transfers to determine if "new" fungus matches original cultures used to inoculate plants.

***Results***

Wilt symptoms should occur on plants inoculated with *Fusarium oxysporum* f. sp. *lycopersici*, but not on plants inoculated with water.

*F. oxysporum* f. sp. *lycopersici* should be recovered from diseased plants but not from control plants. The recovered fungus should be the same as the one used to inoculate the plants.

NOTE: Koch's postulates may also be tested as variations of the following experiments:

A) The Symptoms & Signs of Disease
C) Monoculture & Disease Epidemics
G) Halo Blight of Bean
H) Pectic Enzymes That Destroy Plant Cell Structure

Koch's postulates for bacterial or fungal storage rots of fruits and vegetables can be accomplished in less time than the Fusarium wilt of tomato experiment because the time to grow plants is eliminated.

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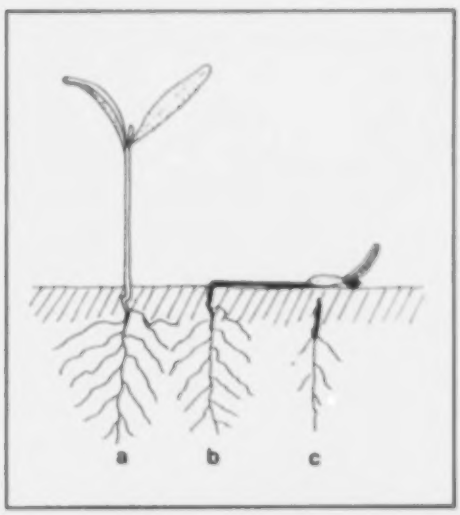
## C. Monoculture & Disease Epidemics

***Introduction***

It is known that some disease epidemics move more rapidly through a population of the same species of plants than through a mixed population. During an epidemic, the rate of disease progress in a monoculture of crop plants can be predicted in order to efficiently manage the disease with a minimum number of fungicide applications. In natural ecosystems disease epidemics are rare unless they result from introduced pathogens against which the native plant has no resistance. This was the case for the chestnut blight that killed American chestnut trees throughout their natural range in North America. Today this tree exists in forests as a shrub, sprouting from the root system, its stems eventually succumbing to the fungal blight.




The fungus *Rhizoctonia solani* causes damping off of seedlings. Damping off is a disease of seedlings characterized by the collapse of the hypocotyl or stem at the soil line, seedlings so affected fall over and die (Figure 8C.1). *Rhizoctonia* will quickly move through a flat of seedlings, killing them in its path (Figure 8C.2). However, disease progress may vary in different mixtures of species of seedlings and the fungus may not kill all the species in a particular mixture.

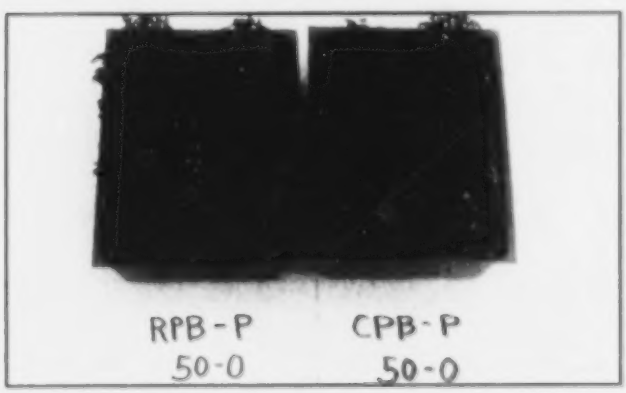



Figure 8C.1. *Damping off of seedlings. The hypocotyl is infected and destroyed, killing the seedling. a) Healthy seedling b) Post-emergent damping off* (i.e. *after seedling emerges from soil) c) Pre-emergent damping off* (i.e. *before seedling emerges from soil).* Figure 8C.2. *Two flats of seedlings showing the effect of damping off.*

***Purpose***

- To study damping off in different mixtures of seedlings.
- To determine the rate of disease progress through uniform and mixed populations.
- To show that disease spread may be reduced or altered in mixed populations of plants.
- To determine the best out of four seedling mixtures for reducing disease incidence and spread.

•To show that disease may spread faster and farther through denser stands of plants.

***Materials***

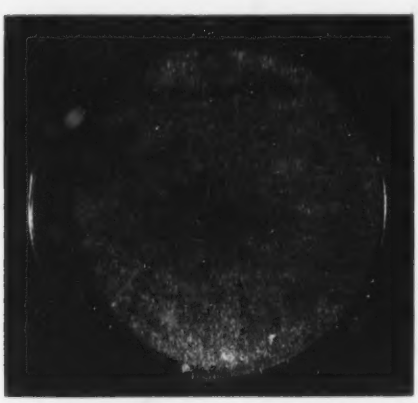
•Four day-old cultures of *Rhizoctonia solani* growing on potato dextrose agar (PDA) plates (Figure 8C.3) (see Chapters 5, 6 and 7).
•Dissecting knives or 0.5 cm diameter cork borers.
•UNTREATED celosia, lettuce, alfalfa and rye seed.
•16 flats (approx. 9 x 12 x 2 inches = 22 x 28 x 5 cm) in which to grow seedlings.
•Sterile potting mix, such as peatlite.
•Plastic or plastic bags.
•Electric heating mat.

***Methods***

Grow *Rhizoctonia solani* at room temperature for four days on 2-4 plates of PDA until mycelium reaches plate edge (see Chapter 7).

Fill the flats with potting mix. Moisten and firm the soil.

In each flat, plant four rows of seed running the width (9 inches or 22 cm) of the flat. Space rows 2 3/4 inches (7 cm) apart with outer two rows 1 3/8 inches (3.5 cm) from edge of flat. Space seed 3/4 inches (2 cm) apart. In one flat plant four rows of only celosia. In another flat, plant celosia alternating with rye within each of the four rows. In another flat, plant celosia alternating with rye then alfalfa within each of the four rows. In another flat plant celosia alternating with rye then alfalfa then lettuce in each of the four rows. Label flats with the treatments. Record the number of seeds of each species placed in each row. Replicate as many flats as desired, but at least two of each so that one will remain uninoculated as a control.



Left: Figure 8C.3. *Culture of* Rhizoctonia solani *growing on potato dextrose agar.* Right: Figure 8C.4. *Microscopic view of* Rhizoctonia solani *hyphae showing characteristic 90 degree angle branching and variable pigmentation.*

Plant another set of flats as described above, but this time space the seed by only 3/8 inch (1 cm) apart. These flats could also be filled in by three more rows of seed planted between the four rows previously described. This would leave only 1 3/8 inch (3.5 cm) between the rows of seedlings. Label flats with the treatments. Record the number of seeds placed in each row.

After sowing seed, inoculate the head or beginning of each row with a plug of *Rhizoctonia* mycelium (Figure 8C.4). With a knife or cork borer cut out a cube (3/8 inch (1 cm) sides) or circle of agar containing a thorough mat of mycelium. Place this into soil at same level as seeds were sown. Try to keep plugs of mycelium uniform in size so that the amount of inoculum per treatment is constant.

Gently sprinkle flats with water. Cover flats with clear plastic or place in plastic bags to keep moist until seeds start to germinate. If possible, place flats on an electric heating mat to provide a soil temperature of 22°F to stimulate germination. Keep flats in warm room to encourage uniform germination rates.

When germination begins, remove plastic and grow seedlings in sunny location for at least 2 weeks. Observe flats for development of damping off symptoms.

Once damping off starts to show up in flats, count number of seedlings killed each day to determine rate of disease progress as expressed in # of seedlings killed/day for each treatment. If seeds do not germinate, this could result from pre-emergence damping off. For pre-emergence damping off, the percent disease incidence can be determined as the number of ungerminated seeds/total number of seeds sown X 100.

After two weeks, count number of each species of seedling still alive and compare to initial number sown to determine best seedling mixture for reducing disease incidence and spread. For damping off, the percent disease incidence can be determined as the number of seedlings with damp off/total number of seedlings germinated X 100.

***Results***

The damping off may be most severe in the monoculture of celosia; less severe in the blend of celosia and rye; less severe in the blend of celosia, rye and alfalfa; and least severe in the blend of celosia, rye, alfalfa and lettuce. However, different results can be obtained. Most monocultures show faster disease progress than mixtures. However, there is evidence that, in some monocultures, conditions may be promoted in the soil that may reduce the incidence of disease caused by certain root-infecting fungi. What plants have you seen that tend to grow in monocultures or colonies in nature?

The denser stands of seedlings should be more severely affected by damping off. Disease progress in these stands may be faster.

***Variations of This Experiment:***
Use a species of the fungus *Pythium*. This fungus also causes damping off and it may be available from Carolina Biological Supply Company or Ward's Natural Science Establishment, Inc. (see Chapter 6 for address).

Another possibility is to use sprout packs from the produce department of your local grocery store. These are available in a variety of "mixtures and monocultures". Choose only those packs in which the roots of the seedlings are white and healthy on the bottom of the pack. Inoculate one corner or edge of the pack near the middle of the hypocotyls of the seedlings; replace the top; and measure the progress of disease.

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## D. Plant Parasitic Nematodes from Soil

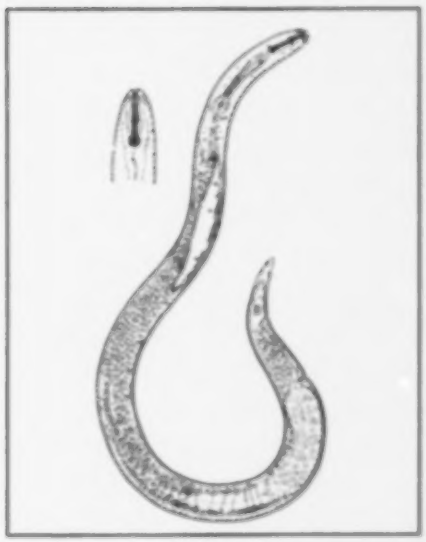


Figure 8D.1. *A nematode in the genus* Hoplotylus. *Its head region (magnified) shows the stylet that is used to feed on and penetrate into plants by plant parasitic nematodes.*

***Introduction***

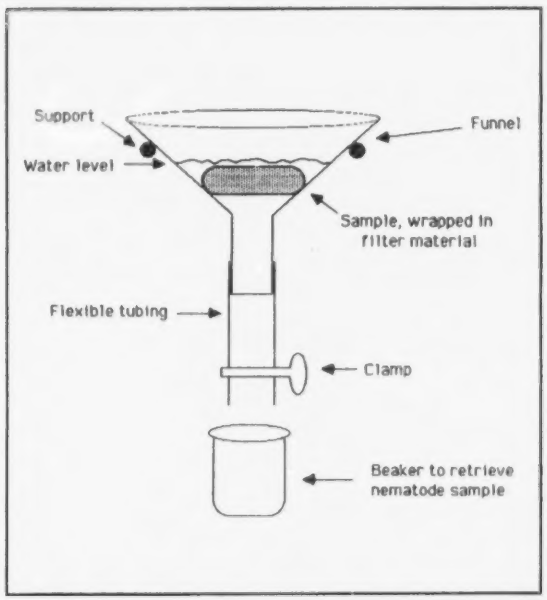
Plant parasitic nematodes are animals belonging to the phylum Nemathelminthes and the class Nematoda (Figure 8D.1). The ways that nematodes can infect plants are diverse: They can parasitize roots, leaves, bulbs, flowers and seed. Natural openings such as stomates in leaves provide entrances, but most break through the outer cells of plant roots by means of their stylets. Nearly all plant parasitic nematodes are soilborne and all have spear-like stylets as their mouthparts to facilitate the breaking of plant cell walls both to gain entrance into the plant and to release nutrients. Crop damage is related to the densities of plant parasitic nematodes in the soil. Therefore, to determine whether or not a crop may be damaged by nematodes, soil samples are collected, the nematodes extracted, and the number of each different type of plant parasitic nematode is counted.

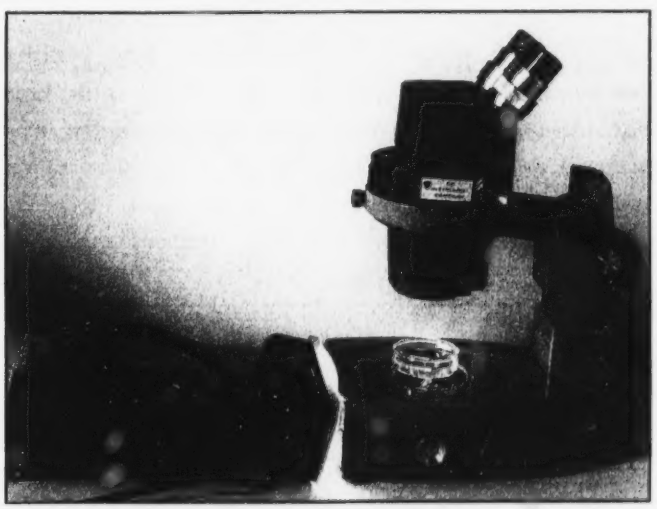
***Purpose***

•To study the nematodes found in natural soils by extracting and observing them.
•To compare stylet-bearing to non-stylet-bearing nematodes.
•To estimate which soil sample contains the greatest number of plant parasitic nematodes and would thus pose the greatest threat of nematode damage and be the least desirable for use as a planting site.

***Materials***

•Plastic bags.
•Trowels.
•100 $cm^3$ (1/2 cup) sample of soil collected from near plant roots from a variety of sources. Label each with the name of the plant or plants growing in the soil the sample was taken from.
•**Baermann Funnel Apparatus** (If possible, have one for each sample of soil.)
Funnels, 10 to 20 cm diameter at top.
Rubber tubing, attached to stem of funnels.
Spring or screw clamps to close off end of tubing farthest from funnel.



Top: Figure 8D.2. *A Baermann funnel for extracting nematodes from soil.* Bottom: Figure 8D.3. *A dissecting microscope with transmitted light source.*

Ring stands with rings to support funnels.
•Milk filters, muslin, cheesecloth, or tissue paper (listed in order of desirability).
•Small beakers (50 or 150 ml), watch glasses, Syracuse dishes or small (60 mm diameter) glass petri dishes.
•Dissecting or stereoscopic microscopes, preferably with stages and glass stage plates to allow transmitted illumination from below.
•Lamps for use with microscopes.
•Compound microscope with light source.
•Microscope slides and cover slips.
•Nail polish (clear is best, but colors will work, too).
•Eyedroppers, glass Pasteur pipets with bulbs or plastic Pasteur pipets.
•Alcohol lamps or small bunsen burners.

***Methods***

Collect soil samples from around plant roots or from different areas in your locality. Each student or pair of students could collect a soil sample. Each sample should consist of about 100 cm3 (1/2 cup) of soil. Place it in a plastic bag and keep it away from heat to protect the nematodes in it. Collect soils that are not compacted. Soil from gardens, parks, forests, lawns, houseplants, fields or orchards will all yield results. Remove large stones and debris from the soil samples.

Prepare Baermann funnels as follows (Figure 8D.2). Attach tubing to funnel stem. The fit should be tight to prevent water leakage. Close off end of tube with clamp. Place funnel in ring support. Adjust height of ring on stand so that end of tubing hangs

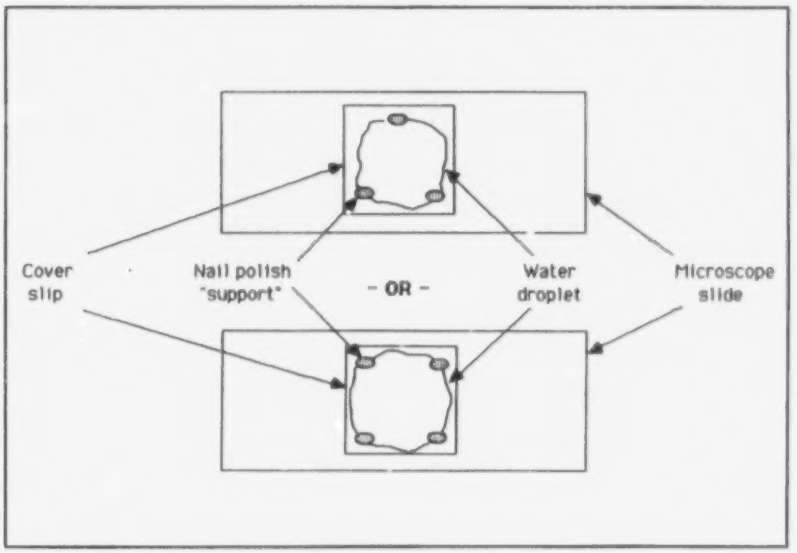



Figure 8D.4. *Arrangement of nail polish drops on microscope slide to facilitate the observation of nematodes.*

free. Allow enough room below tube end to draw water from tubing into small beaker or watch glass. Use a separate Baermann funnel for each soil extraction.

Fill funnel with water to a depth of one inch. Make certain tubing is also filled with water. Place a small (50 to 100 $cm^3$), known volume of soil into a damp milk filter, piece of muslin, or other fine, porous material. Fold filter around soil to enclose it. Fifty to 100 $cm^3$ are roughly equivalent to 50 to 100 ml volume. To facilitate comparisons between soils being extracted, use same amount of soil in each funnel.

Gently place packet of soil into funnel so that it contacts the water in base. Carefully add more water to funnel until soil packet is just submerged. This will allow some oxygen to reach soil sample by diffusion and help keep the nematodes in it viable during extraction. Alternatively, sample packet may be placed in a small plastic or stainless steel strainer that fits inside funnel. The strainer is suspended so that sample is just covered with water. Soil and other debris should remain inside packet, yielding a cleaner sample for the observation of nematodes.

Allow apparatus to stand for several hours or overnight. Then draw off a small quantity of water from tube by briefly opening clamp and collecting run-off into a small beaker or watch glass. Further samples may be taken at several-hour intervals, but keep in mind that after about 48 hours, nematodes at bottom of tube begin to die from lack of oxygen.

Examine the eluted sample using a dissecting microscope, with transmitted light, if possible (Figure 8D.3). This can be best achieved if microscope has a stage with a mirror underneath so that light can be reflected up through a clear glass plate on stage, illuminating sample from below.

Using the highest magnification available, it should be possible to differentiate stylet-

bearing from non-stylet-bearing nematodes. Those that bear stylets are potentially plant parasitic nematodes. For each source of soil, count or estimate numbers of nematodes that have stylets to determine which soil is most infested with potentially plant parasitic nematodes. By estimating numbers of plant parasites in each sample, it should be possible to determine which soil plants would have more nematode problems. If compound microscopes are available, more detailed observations of nematodes can be made.

Take a clean microscope slide and place three or four drops of nail polish on it in a triangular or rectangular pattern. Arrange the drops so that a coverslip will span all of them (Figure 8D.4) and be supported by them, preventing nematodes from being crushed. Using a dropper or pipet, place a small drop of nematode-containing water, as seen under dissecting microscope, on slide inside space defined by drops of nail polish. Carefully pass slide bearing the nematodes over a low flame six to eight times, or until nematodes stop wiggling around in the water droplet. Observe slide under dissecting microscope to ensure that the nematodes have stopped moving. If not, continue to gently heat them until they are completely "heat relaxed." DO NOT OVERHEAT YOUR NEMATODES! Once the nematodes are relaxed, allow slide to cool briefly, then place a coverslip over water drop so that it rests on nail polish supports. If possible, try to avoid trapping air bubbles under coverslip. The nematodes may now be examined under high magnification using compound microscope. Stylets (when present) should be easily visible, along with some details of the nematodes' digestive and reproductive systems (Figure 8D.1).

***Results***

Live nematodes should be recovered from some of the soil samples. Among them stylet-bearing nematodes may be present. Such nematodes are potential plant parasites. In order to determine this, they would need to be identified to genus. However, from the data collected it should be possible to estimate which soil has the greatest potential for nematode problems on the plants being grown there.

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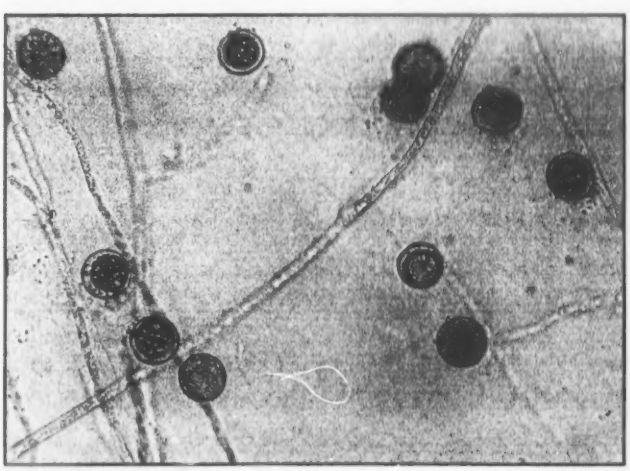
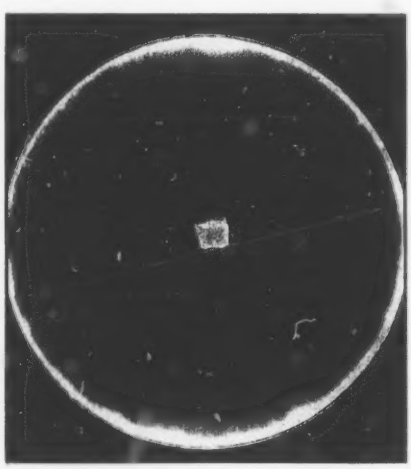
## E. Fungi from Soil

***Introduction***

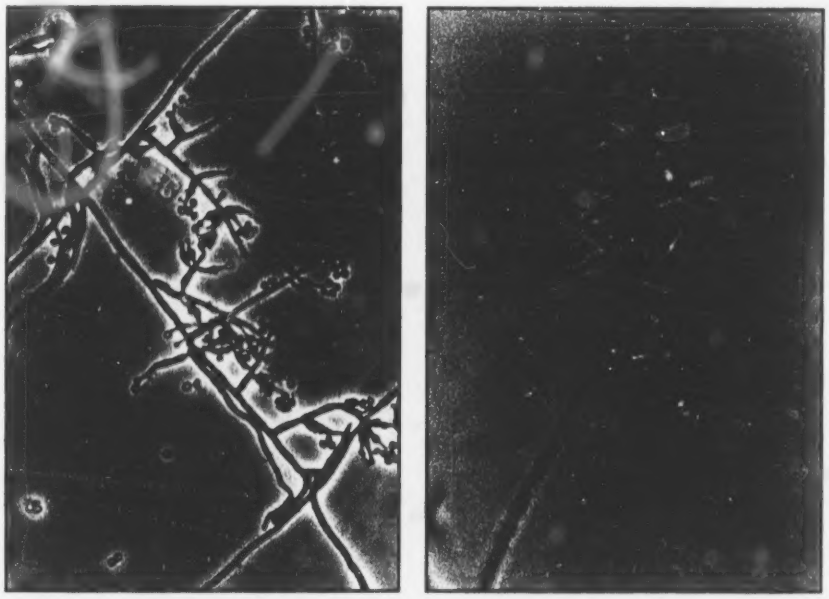
All taxonomic groups of fungi are represented in soil. Most are not pathogenic to plants. However, pathogenic fungi may be isolated from soil by a variety of methods such as using selective media, natural host tissues onto which they will grow (baiting), or soil dilution and plating, to name a few. In this experiment the soil dilution and plating method will be used to attempt to isolate two genera of fungi from soil, *Pythium* spp., pathogens of plants, and *Trichoderma* spp., pathogens of fungi. *Trichoderma*'s are often used for biological control of root, crown and stem rots.

*Pythium* species are rapidly growing fungi whose hyphae (thread-like vegetative bodies) readily grow on agar-containing minimal nutrients. They are members of the Oomycetes or water molds. Some have specific temperature optima for growth: *P. ultimum* 20°C; *P. aphanidermatum* up to 35° C. *Pythium* fungi cause soft, watery rots of seeds, roots, storage organs and crown tissues. *Pythium* hyphae are hyaline (colorless) and aseptate (without cross walls). Their sexual spores, similar to those of some algae, are spherical oospores with thick walls that arise from the fertilization of an oogonium by an antheridium. *Pythium* species also produce sporangia which are variable in shape but often spherical and similar to their oogonia (Figure 8E.1). Oospores and sporangia can either germinate directly to produce hyphae or they can produce zoospores, small spores that are motile by means of two flagella. In culture these fungi will produce colorless to white colonies that are flat to fluffy (Figure 8E.2).





Left: Figure 8E.1. *Sporangia, oospore and aseptate hyphae of* Pythium ultimum *as seen through a compound microscope*. Right: Figure 8E.2. *A culture of* Pythium *sp. on water agar showing sparse and flat growth of hyphae.*



Left: Figure 8E.3. *a)* Trichoderma harzianum *hyphae, conidiophores, and conidia stained with cotton blue.* Right: *b) A conidiophore of* Trichoderma harzianum *with short side branches called phialides from which conidia (spores) are produced.*

*Trichoderma* also grows rapidly on minimal media. These fungi are members of the Deuteromycetes or Imperfect Fungi. Trichodermas attack fungi such as *Rhizoctonia solani* and *Pythium ultimum* by coiling around their hyphae, enzymatically degrading their cell walls, and utilizing their cell contents as a source of nutrition. Trichodermas have hyaline (colorless) hyphae that are septate (with cross-walls) and produce green, hydrophobic masses of conidia (spores) which are unicellular, hyaline and ovoid (Figure 8E.3a). The conidia arise from specialized cells termed phialides grouped on branched conidiophores (Figure 8E.3b). In culture this fungus will grow into a white colony producing small cushions or mounds of conidiophores that are at first white but change to green as spores mature (Figure 8E.4).

***Purpose***

•To study two fungi isolated from soil.
•To learn about fungal morphology and classification.
•To determine the presence or absence of the two fungi in different soils.

***Materials***

•10 g (1/2 cup) each of soil from several sources for comparison (topsoil or any soil from outdoors).
•20 plates of water agar amended with streptomycin sulfate for each source of soil (see Chapter 6).
•Water agar and/or potato dextrose agar plates.
•For each source of soil, sterile water prepared as follows: One 250-ml Erlenmeyer flask containing 90 ml water, capped with aluminum foil and three culture tubes containing 9 ml water, capped with a plastic cap or cotton. Autoclave 20 minutes,

slow exhaust (see Chapter 6).
•A set of five spreading tools for each source of soil, sterile 9-inch long Pasteur pipets: flame tip to seal end and heat an area 1 1/2 - 2 inches up and bend to a 90° angle. Or heat and bend a 1/8 inch or 3/16 inch diameter glass rod in this way. Autoclave or bake to sterilize (see Chapter 6).
NOTE: A single spreading tool/soil source may be used, but it must be dipped in ethanol, flamed, and cooled prior to use for spreading the different soil dilutions.
•Sterile 1 ml pipets (graduated to 0.01 ml).
•Pipet bulbs.
•Thiabendazole (Sigma T8904).
•Ethanol, 95%.
•Compound microscopes, slides and coverslips.

***Methods***
Read the safety information inChapter 4 on ethanol and thiabendazole. All other chemicals pose little hazard, but always read the chemical label on the container.

Have each student bring in about 1/2 cup of soil from a garden, park, lawn, houseplant, forest, etc. Avoid rocks, gravel, sticks or large roots.

Label different sources of soil.

Prepare soil suspensions: To each flask containing 90 ml sterile water, add 10 g of soil from one source and label flask accordingly. Swirl to mix and suspend soil thoroughly in water. This is the 1:10 (weight:volume) dilution.

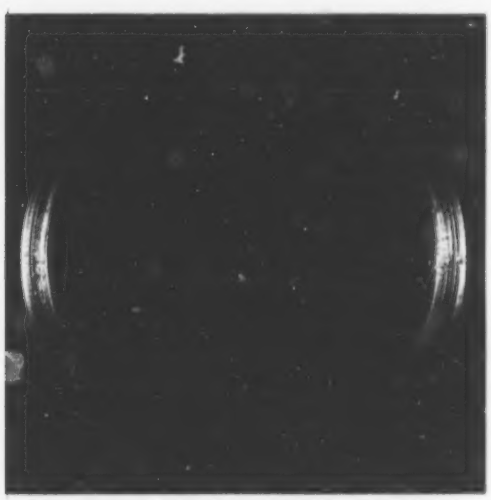


Figure 8E.4. *A culture of* Trichoderma *sp. on potato dextrose agar showing fluffy, dense hyphae and green coloration from the conidia (spores).*

Prepare dilution series:
Proceed for each source of soil, start with the 1:10 dilution, and keep all dilutions labeled as to source of soil.

Place three 9-ml sterile water blanks in a rack. Label one '1:100', the second '1:1,000', and the third '1:10,000'. Label each set with soil source.

For 1:100 —with a sterile 1-ml pipet, remove 1 ml of the 1:10 soil suspension and place it in 1:100 tube. Mix thoroughly by pipeting up and down and cap. Or cap and

vortex thoroughly.

For 1:1,000—with a sterile 1-ml pipet, remove 1 ml of the 1:100 dilution and place it in 1:1,000 tube. Mix as above.

For 1:10,000—with a sterile 1-ml pipet, remove 1 ml of the 1:1,000 dilution and place it in 1:10,000 tube. Mix as above.

Spread soil dilutions onto water agar with streptomycin plates as follows:
Proceed for each source of soil and its dilutions. Label plates with the source of soil and the dilution. The streptomycin in the water agar will inhibit the growth of bacteria found in the soil and enhance the recovery of fungi.

For Trichoderma - With sterile 1 ml pipet, remove 0.1 ml of 1:100 dilution and drop it onto the surface of the agar in the center of the plate. Repeat this on at least one more plate to replicate the cultures from each source of soil. With sterile spreading tool, spread liquid thoroughly over surface of the two agar plates. Do the same with the 1:1,000 and 1:10,000 dilutions. Always use sterile spreading tools for each dilution.

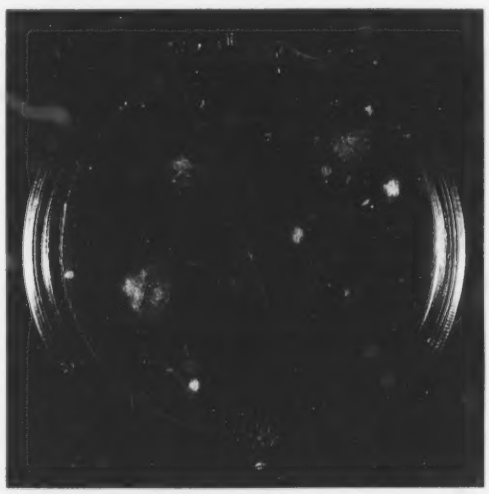


Figure 8E.5. *A soil dilution plate with several fungal colonies growing on it, including some of* Trichoderma.

Prepare 100 ml of stock solution of thiabendazole in 95% ethanol at 10 mg/ml. To 100 ml 95% ethanol, add 1g of thiabendazole. Place in screw cap bottle and mix to dissolve and suspend thoroughly. Thiabendazole may not dissolve completely in ethanol;prior to use, suspend thoroughly. 100 ml of stock is enough for 80 to 90 sources of soil.

For Pythium - AFTER spreading dilutions on the 'Trichoderma' plates, with 1 ml pipet add 0.5 ml thiabendazole stock solution to each of the flasks and 0.05 ml to each of the 1:100 dilution tubes. Mix well. This yields a 50 ppm concentration of thiabendazole. The thiabendazole will inhibit many fungi, including *Trichoderma*, but not *Pythium*. Allow soil to settle in flask. With a sterile 1-ml pipet, remove 0.5 ml of the 1:10 dilution (in flask) and drop it onto the agar. Repeat this on a second plate to replicate the cultures. Spread liquid as described above. Remove 0.5 ml of the 1:100 dilution and spread this on a plate, replicating once as described.

Incubate these plates at room temperature for 2-3 days and observe daily under the dissecting microscope (Figure 8E.5).

To obtain pure cultures of the fungal colonies that grow on the plates, transfer the tips of hyphae from the edge of the colony to a fresh plate of water agar amended with streptomycin. After the fungus grows for 2 to 3 days, transfer hyphal tips to water agar or potato dextrose agar (if available) as described in Chapter 7.

***Results***

*Pythium* will grow rapidly out of soil onto the water agar. Its hyphae are sparse and hyaline or colorless. Angle the plates into a light source to see the sparse hyphal growth. Place a coverslip over the agar on top of the hyphae and examine directly on the stage of a compound microscope.

*Trichoderma* will also grow out rapidly onto the water agar. Its hyphae are more densely produced and will give rise to the small cottony tufts that later turn green as spores mature. When green pigmentation is observed, the fungus may be examined by mounting tiny bits of mycelia on a glass slide in water plus Tween 20, a surfactant (1 drop/100 ml), covering with a coverslip, and observing under a compound microscope. The Tween 20 helps overcome the hydrophobic nature of *Trichoderma's* spores. Any liquid detergent may be used for this.

Many other fungi may be recovered using these methods in addition to *Pythium* and *Trichoderma*. These may be compared microscopically to the other two. *Pythium* may not be recovered from some soils, but *Trichoderma* is very common and should be readily recovered.

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## F. Classification of the Powdery Mildews

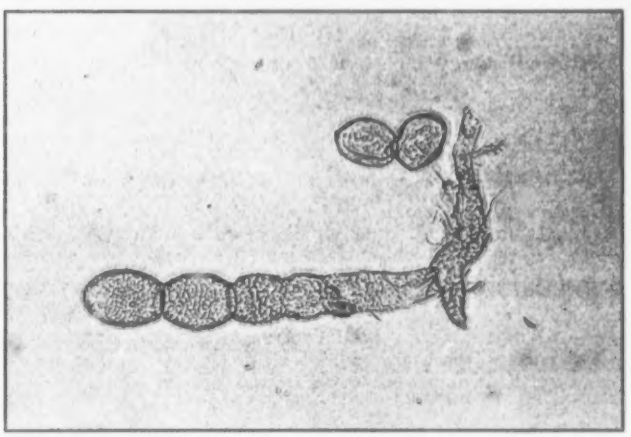
***Introduction***

Powdery mildews are obligate parasites of plants: They cannot be grown on artificial culture media and require living plant cells to grow. These fungi reproduce by means of two spore types: asexual spores (conidia) and sexual spores (ascospores). Conidia are barrel shaped and arise in chains from the mycelium or 'mildew' growing on the plant's leaves, stems or flowers (Figure 8F.1). Ascospores are oval and are born in sacs called asci (sing. ascus). The sacs or asci are contained in tiny, round, black cleistothecia (Figure 8F.2). Cleistothecia are ascospore-bearing structures characterized by being fully closed. Other ascospore-bearing structures produced by fungi are characterized by being fully open or cup shaped (apothecia) or opening through a pore in the top (perithecia). The cleistothecia produced by powdery mildew fungi are formed on the surface of the mycelium in late summer and autumn. Cleistothecia have appendages that radiate out from their outer surface. Inside the cleistothecia may be found either a single ascus or many asci. The powdery mildews are classified to genus based on the number of asci contained in the cleistothecia and on the morphology of their appendages.

***Purpose***

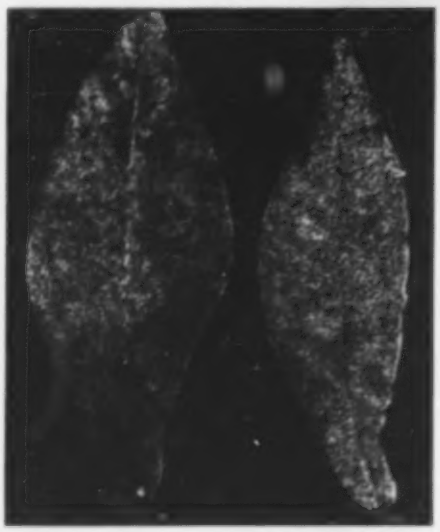
- To study powdery mildew diseases of plants.
- To identify powdery mildew fungi and learn about fungal classification.
- To learn about the types of reproductive spores in fungi.

Right: Figure 8F.1. *Barrel-shaped conidia (asexual spores) produced in chains, as seen through a compound microscope.* Below: Figure 8F.2. *Cleistothecium broken open to reveal ascospores (sexual spores) within asci, as seen through a compound microscope.*



***Materials***

•Plant leaves with powdery mildew. (The powdery mildews that occur on plants are not allergenic.)
•Water in dropper bottles.
•Binocular dissecting microscopes.
•Compound microscopes.
•Dissecting needles.
•Micropipets or Pasteur pipets and bulbs.
•Microscope slides and coverslips.
•Hot plate.
•Large beaker (800 ml).
•Piece of screen to fit in the beaker.
•Tongs or forceps.
•Envelopes.



Top: Figure 8F.3. *a) Powdery mildew on garden Phlox.* Opposite: Figure 8F.3. *b) Close-up of two leaves showing black freckles that are the cleistothecia.* Bottom: Figure 8F.4. *Oak leaf with powdery mildew containing the tiny cleistothecia that appear as black dots, as seen through a dissecting microscope.*

***Methods***

Collect plant leaves in late summer or autumn that show signs of powdery mildew and cleistothecia. Lilac, phlox, rose, wheat, lawn grasses, grape, oak, horse-chestnut, and many other shrubs, trees, flowers, weeds and garden plants are susceptible to powdery mildew. Look for pale, dusty-white coatings on leaves (Figure 8F.3a). With a hand lens or magnifying glass examine the fine threads of the mildew for round, black cleistothecia (Figure 8F.3b).

Leaves that have good specimens of powdery mildew on them can be preserved in a plant press and herbarium and saved and reexamined over many years. Prepare a label on paper to be stored with pressed leaves giving the plant name, date of collection, location of collection and name of collector.

When collecting plant leaves on field trips, always be aware of the poisonous or rash-producing plants in your area and take precautions to avoid them. The powdery mildews that occur on plants are not allergenic.

In the classroom, choose a leaf containing cleistothecia (Figure 8F.4) and examine with dissecting microscope.

If leaves are dry, steam to soften. On a hot plate boil a small amount of water in a beaker with a screen suspended over water. Place dry leaves in open envelope and, using tongs or forceps, place this over screen to steam for 10 minutes. Use tongs or forceps to remove leaves from steam. Wear goggles when working over boiling water.

Place a drop of water on a group of cleistothecia seen under the microscope. Wait one minute.

While looking through the microscope, very gently tease the cleistothecia off of the mycelium into the water drop with a dissecting needle so as not to break their appendages.

Touch the surface of a microscope slide to the drop of water on the leaf. Quickly flip slide right side up and cover the drop of water with a coverslip. Alternatively, working under the dissecting microscope, suck up cleistothecia with a micropipet or a Pasteur pipet and place them onto a slide and cover with a coverslip.

Examine drop on slide with compound microscope.

Look for appendages on the cleistothecia and compare them to the illustrated key (Figure 8F.5).

Determine how many asci are in the cleistothecia by gently pressing down on coverslip with eraser end of a pencil to break them open. Mature cleistothecia will contain asci with ascospores; when immature they will contain amorphous material.

Use the illustrated key (Figure 8F.5) to identify the powdery mildew to genus.

Repeat for other powdery mildews collected. Credit can be earned as specimens are collected, classified and preserved. Drawings of the cleistothecial characters can be prepared for verification of the identifications made.

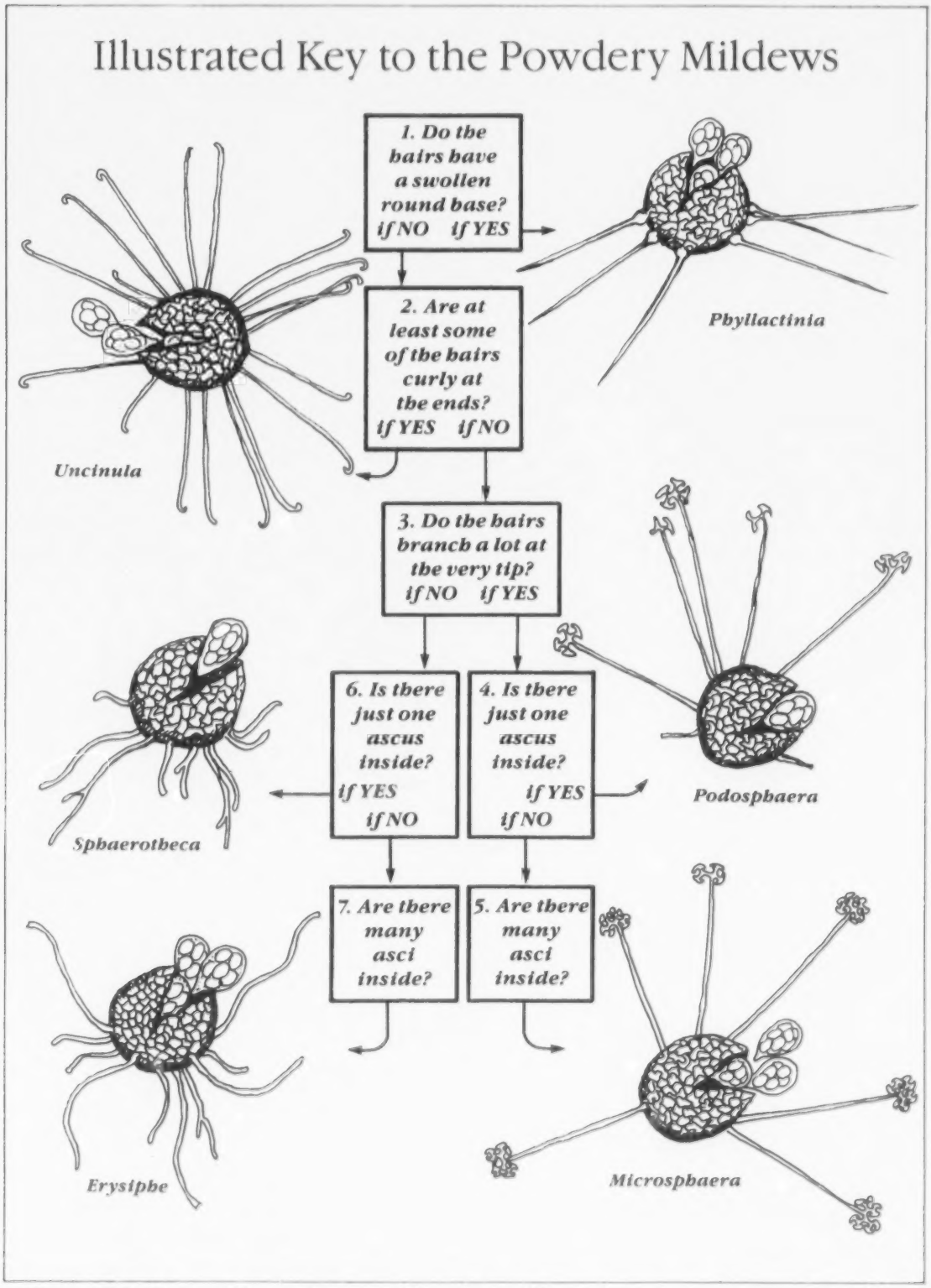

Illustrated Key to the Powdery Mildews
1. Do the hairs have a swollen round base? if NO if YES
2. Are at least some of the hairs curly at the ends? if YES if NO
3. Do the hairs branch a lot at the very tip? if NO if YES
6. Is there just one ascus inside? if YES if NO
4. Is there just one ascus inside? if YES if NO
7. Are there many asci inside?
5. Are there many asci inside?
Uncinula
Phyllactinia
Podosphaera
Sphaerotheca
Erysiphe
Microsphaera

Figure 8F.5. *Illustrated key to the genera of powdery mildews.*

***Results***

Powdery mildews in a variety of genera should be identified. An appreciation of the means of classification of fungi should be gained.

***References***

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## G. Halo Blight of Bean

***Introduction***

Halo blight is a destructive bacterial disease of bean infecting leaves, flowers, pods and stems. Seeds can become infected and carry bacteria to new plantings. The disease is caused by *Pseudomonas syringae* pathovar *phaseolicola*. *P. s.* pv. *phaseolicola* is a fluorescent pseudomonad. Cultures grown on King's medium B will fluoresce under long wave ultraviolet light. The disease's common name is halo blight because in nature infections of leaves appear as small brown spots with large, lemon-yellow halos around them (Figure 8G.1). Halos develop on leaves in response to a toxin, phaseolotoxin, that diffuses into tissues in advance of the bacteria producing it. In very severe infections, systemic chlorosis or yellowing can occur, rendering the younger plant tissue stunted and chlorotic due to systemic spread of the toxin. It has been shown that it is the production of phaseolotoxin by the bacteria that causes the appearance of the chlorotic haloes and systemic chlorosis, not systemic spread of the bacteria. When only purified phaseolotoxin is applied to leaves it will cause stunting and chlorosis.

The mode of action of phaseolotoxin is to inhibit the activity of the enzyme ornithine carbamoyltransferase (OCTase) that is involved in the biosynthesis of the amino acid arginine. Amino acids are the building blocks of proteins. OCTase inhibition results in the accumulation of ammonia in plant tissues. The inhibition of arginine synthesis probably leads to the stunting of plants. While the accumulation of ammonia probably causes the chlorosis.

The inhibitory activity of phaseolotoxin is specific for the enzyme OCTase. But since all plants, and in fact most organisms, are somewhat dependent on OCTase for proper growth, the toxin is nonspecific and can be inhibitory to most organisms--certainly most plants. Because the toxin is nonspecific with respect to organism, its activity can be assayed against the bacterium *Escherichia coli*. The assay is carried out on minimal medium (M9) because the toxin's

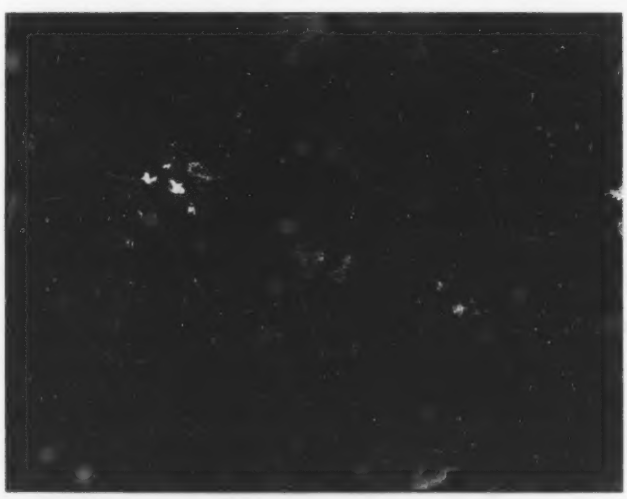


Figure 8G.1. *Halo blight on bean leaves. Note large halos around brown spots.*

effects are observed as a result of arginine depletion. Richer media, such as nutrient agar or King's medium B, that contain amino acids, would provide our indicator organism, *E. coli*, with arginine, negating the effects of OCTase inhibition by the toxin. Phaseolotoxin poses no risk to humans handling *P. s.* pv. *phaseolicola*. An interesting question for scientists remains unanswered: How does *P. s.* pv. *phaseolicola* protect itself from phaseolotoxin?

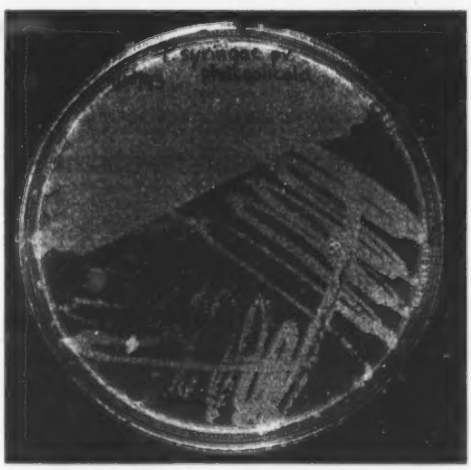
PART A - Halo Blight of Bean

***Purpose***

•To study a bacterial disease of bean.
•To study fluorescent bacteria and fluorescence as a means of classifying bacteria.
•To induce chlorosis in plants in response to phaseolotoxin.

***Materials***

•Pinto or red kidney bean plants grown to the first or second set of trifoliate leaves (about 3 weeks old).
•Plastic bags large enough to enclose the bean plants. Clean plastic garbage pails may also be used.
•Sterile mist sprayers or atomizers: Sterilize by soaking for 10 minutes in 10% bleach, rinsing in sterile distilled water and air drying; if glass, by autoclaving.
•Sterile water.
•Cultures of *Pseudomonas syringae* pv. *phaseolicola* growing on nutrient agar plates (see Chapters 5 and 7).
•Inoculating loops and dissecting needles.
•Bent glass rod or rubber policeman, sterile.
•Needlepoint flower holder (Figure 8G.2): Clean and sterilize by soaking in a 10% bleach solution for 10 minutes, rinsing in sterile water and air drying to dissipate the chlorine.
•Cheesecloth squares (4 x 4 in. or 10 x 10 cm).
•Cardboard squares, same size as cheesecloth.
•Nutrient agar plates (see Chapter 6).



Top: Figure 8G.2. *Needlepoint flower holder.*
Bottom: Figure 8G.3. *24-48 hour culture of* Pseudomonas syringae *pv.* phaseolicola *on King's medium B.*

•King's medium B plates (see Chapter 6).
•Long wave UV light (black light). Long wave UV light is safe for eyes. It is useful to tack the light to the outside of the bottom of a cardboard box. Cut 2 holes in the bottom: one for the light to shine through and one to look through and invert the box over the plates. This way the UV light is contained in the box, ambient light is excluded, and the plates are more easily viewed.

***Methods***
Read the safety information in Chapter 4 on bleach. All other chemicals pose little hazard, but always read the chemical label on the container.

Grow bean plants to the first or second set of trifoliate leaves. Plants should have leaves that are 1/3 to 2/3 expanded for inoculation.

With an inoculating loop and using sterile technique (see Chapter 7), transfer single colonies of *P. s.* pv. *phaseolicola* from nutrient agar to 2 or 3 King's B plates. Grow for 48 hours at room temperature (Figure 8G.3). After allowing for bacterial growth, place plates under long wave UV light to check for fluorescent colonies.

Using sterile technique, transfer cells from single, isolated fluorescent colonies to nutrient agar plates and grow for 24-48 hrs at room temperature. Pour sterile water over the 24-48 hour cultures and using sterile rubber policeman or bent glass rod gently rub bacteria off surface of agar to suspend them in the water. Avoid gouging chunks out of the agar.

Using one of the methods described below, mock-inoculate one plant's leaves with sterile water without bacteria in order to observe the response of plants to the inoculation method in the absence of bacteria.

If a needlepoint holder is available, pour the bacterial suspension onto a square of cheesecloth resting on a piece of cardboard. Inoculate YOUNG bean leaves that are about 1/3 - 2/3 expanded by gently pressing a leaf between the needlepoint holder from above and the cheesecloth inoculum backed by cardboard (to prevent puncturing hands) from below. Then carefully pull away from the leaf. Repeat for all other leaves to be inoculated. It is best to make small punctures through the leaves into the cheesecloth using gentle pressure and avoid puncturing the cardboard. The prongs of the needlepoint holder will provide wounds through which the bacteria can readily enter and infect the leaves.

If a needlepoint holder is unavailable, pour the suspensions of bacteria into the atomizers or mist sprayers. With the dissecting needles, poke tiny holes in the young

leaves of a few of the plants from above while supporting the leaf from below with cardboard. After puncturing, spray the bacterial suspensions onto the leaves. Or alternately, pour the inoculum onto cheesecloth backed by cardboard and hold this against the bottom of a leaf while poking tiny holes in the leaf from above making sure the cheesecloth is contacted by the needle. The different inoculation techniques may be compared.

All inoculated leaves should be 1/3 to 2/3 expanded, as fully expanded leaves are not susceptible.

After inoculation, water the soil and enclose plants in plastic bags or pails for 24 hours to provide an environment with high relative humidity. After 24 hours, remove plants from the enclosures. To prevent wilting, acclimate them at low humidity, low light for 1 to 2 hours before placing them in a sunny location. Keep the plants in a sunny location and observe weekly for the development of symptoms. If after 7 days no water-soaked lesions have developed, return the plants to plastic bags or pails after thoroughly misting them with water.

To induce the formation of chlorotic halos around the lesions, if possible, place some plants outdoors in a location receiving full sun where the temperature is between 18 - 26°C or in a greenhouse or near a window receiving as much light as possible. Light may be supplemented indoors with incandescent or fluorescent lighting.

***Results***

Inoculated plants will develop small watersoaked lesions on the susceptible leaves that will eventually become necrotic. Plants grown under low light intensity and cool temperatures will not develop halos around the spots because the toxin is not produced by the bacteria in plants under these conditions.

*P. s.* pv. *phaseolicola* can be easily recovered from the water-soaked lesions. Using sterile technique, excise a water-soaked lesion and smear it over the surface of King's B agar and look for fluorescent colonies after 48 hours of growth. To identify the colonies recovered, assay for pathogenicity to bean and for phaseolotoxin production (described below).

PART B - Phaseolotoxin Assay

***Purpose***

To run a microbial assay for phaseolotoxin.

***Materials***

•Cultures of *Pseudomonas syringae* pv. *phaseolicola* growing on nutrient agar or

King's B (Figure 8G.3) (see Chapters 5 and 7).
•Minimal media M9 plates (see below).
•1 M magnesium sulfate ($MgSO_4 \cdot 7H_2O$) solution, sterilized by autoclaving.
•100 mM calcium chloride ($CaCl_2 \cdot 2H_2O$) solution, sterilized by autoclaving.
•Cultures of *Escherichia coli* strain K-12 growing on nutrient agar (see Chapters 5 and 7).
•Test tube racks.
•Sterile water blanks, about 10 ml in each (see Chapter 6).
•Overlay agar: Sterile tubes of 1% water agar, 7 ml in each (see Chapter 6).
•Sterile 1 ml and 10 ml pipets.
•Pipet bulbs.
•55°C water bath - a hot plate with a beaker 1/2 full of water maintained at 55°C using a thermometer will work.
•Vortex.

***Methods***

Read the safety information in Chapter 6 on ammonium chloride, calcium chloride, magnesium sulfate, sodium phosphate, and potassium phosphate monobasic. All other chemicals pose little hazard, but always read the chemical label on the container.

**Prepare minimal media M9 plates:**

Prepare magnesium sulfate and calcium chloride stock solutions:

**1M magnesium sulfate** - Place 30 ml distilled water in a beaker and add 12.3 g of $MgSO_4 \cdot 7H_2O$ (magnesium sulfate with 7 waters of hydration). Using a magnetic stir bar, stir until dissolved on a stir plate. Pour solution into 50 ml graduated cylinder and bring volume up to 50 ml by adding distilled water. Pour solution into autoclaveable 100 ml (or larger) capacity screw cap bottle. Cap loosely.

**100 mM calcium chloride** - Place 95 ml distilled water in a beaker and add 1.5 g of $CaCl_2 \cdot 2H_2O$ (calcium chloride with 2 waters of hydration). Using a magnetic stir bar, stir until dissolved on a stir plate. Pour solution into 100 ml graduated cylinder and bring volume up to 100 ml by adding distilled water. Pour solution into autoclaveable 200 ml (or larger) capacity screw cap bottle. Cap loosely.

Autoclave stock solutions 20 minutes, slow exhaust (see Chapter 6). Remove from autoclave, tighten caps, and allow to cool. Use stock solutions with sterile technique. Store at room temperature or in the refrigerator.

**Salts solution** - In a 1-liter flask place a magnetic stir bar, 450 ml distilled water and add the following salts:

| | |
|---|---|
| Sodium phosphate, $Na_2HPO_4$ | 6 g |
| Potassium phosphate monobasic, $KH_2PO_4$ | 3 g |
| Sodium chloride, NaCl | 0.5 g |
| Ammonium chloride, $NH_4Cl$ | 1 g |

On a stir plate stir until salts are dissolved. Pour solution into 500 ml graduated cylinder and bring volume up to 500 ml with distilled water. Pour solution back into 1 liter flask and cap with foil.

**Agar solution** - In another 1-liter flask place a magnetic stir bar, 500 ml distilled water and add the following:

| | |
|---|---|
| Agar | 20 g |
| Glucose or sucrose | 2 g |

On a stir-hot plate, stir and heat to dissolve sugar and most of agar. (Agar will dissolve during autoclaving.) Remove from heat and cap flask with foil.

Autoclave both solutions 20 minutes, slow exhaust (see Chapter 6). After autoclaving, allow solutions to cool enough to handle. Pour the salts solution into the agar solution. Replace foil cap. Place on a stir plate and stir to mix.

USING STERILE TECHNIQUE and sterile pipets, add to the cooled flask of media:

| | |
|---|---|
| 1M magnesium sulfate solution, sterile | 1 ml |
| 100 mM calcium chloride solution, sterile | 1 ml |

Place on a stir plate and stir to mix. Pour plates. Allow agar in plates to solidify at room temperature and then invert to prevent condensation on lids.

Using sterile technique, streak *P.s.* pv. *phaseolicola* onto M9 plates to make certain your isolate grows on this medium. It should be slightly fluorescent on M9 (see Chapter 7).

Using sterile technique, streak *E. coli* onto M9 plates to make certain your isolate grows on this medium (see Chapter 7). (If *E. coli* isolate will not grow on M9, try another *E. coli* isolate, or use *Erwinia carotovora* from the Pectic Enzymes experiment.) Grow for two days and transfer again by streaking onto fresh M9 plates. Streak out one or two plates. One plate will yield enough for 100 overlays. Grow these plates for two days at room temperature.

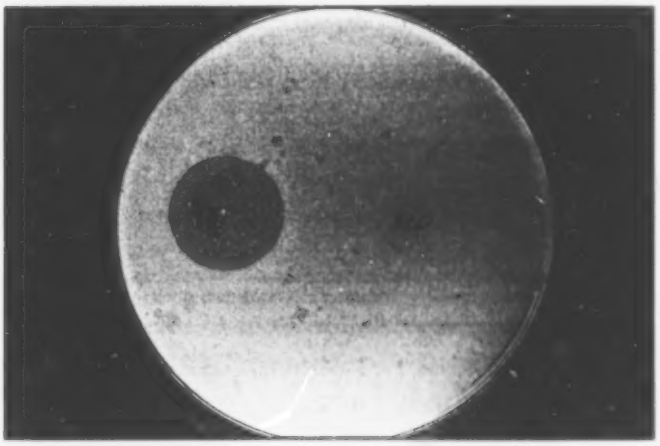


Figure 8G.4. *M9 assay plate showing zones of inhibition over* Pseudomonas syringae *pv.* phaseolicola *colonies.*

Pour 10 ml sterile water onto each *E. coli* plate and gently remove and suspend the bacteria with a sterile rubber policeman or bent glass rod. Carefully pour the suspension back into the tube. Store *E. coli* suspension in refrigerator. The cells can be kept this way for several days and subsequently used in the assay.

Prepare the M9 assay plates as follows. Using sterile technique, spot *P. s.* pv. *phaseolicola* onto an M9 plate by picking up some cells with an inoculating loop and touching this to the surface of the agar. Spot a good amount, so you can see a visible spot of bacteria--about the size of a pinhead. Place the spots of bacteria in a grid pattern so that they are easier to keep track of. If possible, also spot some other bacteria on the plates (such as *E. coli, Agrobacterium tumefaciens* - Experiment J, or *Erwinia carotovora* -Experiment H). Keep track of where they are. Label the bottom of the plate by writing the initials of the bacterium's name on the location of each of their spots. Invert plates and incubate at room temperature overnight.

The following day, melt overlay agar (1 tube/assay plate) by loosening caps and heating tubes in a microwave on high for 1 to 3 minutes or in a beaker of water brought to boiling on a hot plate. Hold tubes of melted overlay agar in a 55°C water bath and allow to cool to 55°C. Take suspensions of *E. coli* out of refrigerator and allow to warm to room temperature.

When the overlay tubes are at 55°C and *E. coli* suspensions are at room temperature, vortex *E. coli* suspension and with a sterile 1-ml pipet transfer 0.1 ml of the turbid *E. coli* suspension to the agar and vortex agar. Don't waste any time or agar may harden. Pour the entire *E. coli* overlay onto the surface of the spotted assay plate, and let it sit right side up until the overlay agar hardens. Repeat *E. coli* overlay onto additional assay plates prepared.

Incubate the overlayed plates at 37°C or room temperature. Growth of the indicator organism, *E. coli* in this case, will result in the overlay agar becoming increasingly opaque. Toxins or other inhibitory compounds, if secreted by the bacteria spotted

onto the plate under the overlay, will have diffused into the agar medium and the overlay, resulting in a clear area in the agar overlay (Figure 8G.4). The size of this "zone of inhibition" can be directly related to 1) the amount of antimicrobial compound produced and 2) whether or not it is secreted by the source bacterium. At 37°C, zones should become visible in 6 hours, maybe less. At room temperature, zones should become visible the following day.

Measure the zones of inhibition in the overlay agar for each of the assayed colonies. Do any of the other bacteria spotted on the plates produce toxins that inhibit the growth of *E. coli*? Do any of the colonies of *P. s.* pv. *phaseolicola* produce more toxin than others? Larger zones of inhibition could result from larger colonies with more bacterial cells.

***Results***

Zones of inhibition should be observed over the colonies of the halo blight bacteria. No zones of inhibition should occur over colonies of *E. coli, Agrobacterium tumefaciens* from experiment J, *Erwinia carotovora* from experiment H, or colonies of saprophytic fluorescent Pseudomonads.

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## H. Pectic Enzymes That Destroy Plant Cell Structure

***Introduction***

Plant cells are surrounded by cell walls. Studies into how bacteria cause soft rot and death of cells elucidated the function and biochemistry of the cell wall and middle lamella. The function of the cell wall is to contain the turgid protoplast of the plant cell, preventing its rupture and cell death. The function of the middle lamella is to hold cells together in tissues. Cell walls consist of densely packed cellulose fibrils cemented by a matrix of hemicellulose, pectin and extensin. Pectin is the first cell wall constituent encountered by microbial enzymes that degrade cell walls. Pectin consists of pectic polymers made up of a backbone chain of galacturonic acid residues. Some bacterial and fungal plant pathogens produce pectic enzymes. Pectic enzymes cleave the $\alpha$ 1,4 linkage between the galacturonic acid residues of the polymer. In a plant tissue, the pectic polymers are especially important because, in addition to being a cell wall constituent, they are the glue in the middle lamella that holds cells together. Thus, pectic enzymes are one of the mechanisms that plant pathogens use (1) to gain entry into plant tissues by degrading the middle lamella and macerating the tissue and (2) to feed on the nutrients released from protoplasts as they rupture due to the loss of integrity of their cell walls. The result is a soft, liquidy rot.

The bacteria that cause soft rots of plants are primarily found in two genera: *Erwinia* and *Pseudomonas*. To verify that an isolate is a soft rot bacterium, a soft rot assay is performed using potato or carrot slices, Belgian endive leaves, or Chinese cabbage midribs. This assay can also be used to measure differences in virulence of different isolates of soft rotting bacteria by measuring differences in the extent of maceration among the isolates. In this experiment a soft rot assay will be performed using *Erwinia carotovora* subspecies *carotovora*. These soft rot bacteria secrete pectic enzymes in culture and in infected tissue. In addition, enzyme assays will be run to determine if the enzymes alone can cause the symptoms of soft rot in plant tissue.

PART A - Soft Rot Bioassay

***Purpose***

- To learn the constituents of plant cell walls and the role pectin plays in the integrity of cell walls and plant tissues.
- To learn one of the causes of the breakdown of plant tissue into a soft, liquidy mass.

***Materials***

- 24 hour cultures of *Erwinia carotovora* subsp. *carotovora* (Figure 8H.1) grown on nutrient agar (see Chapters 5, 6 and 7). Or soft rot bacterial isolates from rotting

Figure 8H.1. *24-48 hour culture of* Erwinia carotovora *sbsp.* carotovora *on nutrient agar.*

produce (cucumber, potato, green pepper). See end of experiment for method of isolating bacteria from plants.
•Inoculating needles and loops.
•Forceps.
•Knives or scalpels.
•Indelible marker.
•Ethanol, 95%.
•Ethanol, 70%.
•Bunsen burners or alcohol lamps.
•Sterile water blanks.
•Sterile petri plates.
•Carrots, potatoes, Belgian endive or Chinese cabbage.

***Methods***

Read the safety information in Chapter 4 on ethanol. All other chemicals pose little hazard, but always read the chemical label on the container.

Peel carrots and potatoes. Wash peeled carrots and potatoes, Belgian endive, and Chinese cabbage leaves in tap water. Cut carrots and potatoes into slices 7 mm (1/4 inch) thick. Cut cabbage leaf blades off of the wide, flat midrib. Cut midrib into 6-cm long pieces.

Surface sterilize the work surface by wiping it with 70% ethanol.

Open sterile petri plate lid slightly and pour sterile water into a depth of about 4 mm (1/8 inch) for carrots or potatoes and 2 mm (1/16 inch) for Belgian endive and Chinese cabbage. Replace lid. Place Belgian endive leaves into plates (1 leaf/plate). With forceps pick up vegetable pieces and dip in 95% ethanol. Drain excess ethanol and pass piece briefly over flame to ignite ethanol. Allow ethanol to burn off. This surface sterilizes the tissue piece. Immediately place vegetable piece into petri plate containing sterile water: 4 to 5/plate for carrot slices, 1/plate for potato slices, and 1/plate for cabbage midribs (Figure 8H.2).

Inoculation - carrots and potatoes: Using sterile technique, with knife or scalpel gouge small (2 mm) notch out of center of each carrot or potato slice. With a flamed

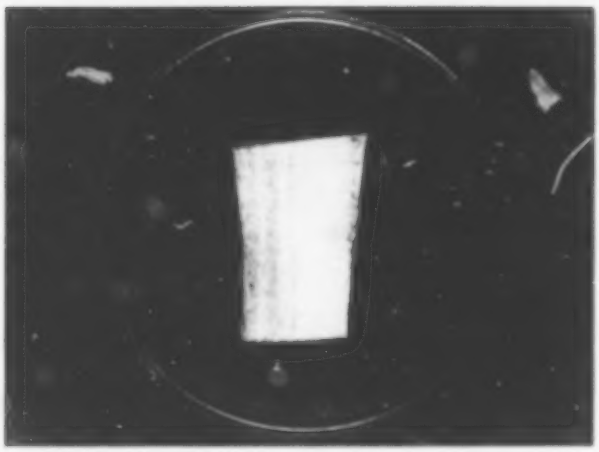

Figure 8H.2. *Cabbage midrib in plate ready for inoculation.*

and cool loop glide over plate of bacterial colonies and pick up bacterial cells. Inoculate notch with loop of bacteria. Replace lid and label plate with isolate used for inoculation.

Inoculation - Belgian endive and Chinese cabbage: With sterile dissecting needle pick up an isolated bacterial colony and stab it into center of midrib. Replace lid and label plate with isolate used for inoculation.

Leave pieces of each vegetable uninoculated to serve as controls. If available, inoculate pieces with single, well separated colonies of bacteria you have isolated.

Incubate plates at room temperature for 48 hours. Test for soft rot by probing tissue with loop, needle or spatula. Soft rotted tissue will slough easily (Figure 8H.3). The extent of soft rot may be visible. If so, measure the diameter (carrot and potato slices) or length (midribs) of the soft rotted tissue to compare the virulence of different soft rot isolates. The faster soft rot progresses, the more virulent the isolate or the more concentrated the dose of bacterial inoculum.

***Results***

Soft rot will occur in the plant tissues inoculated with soft rot bacteria as tissue is macerated by the pectic enzymes produced by these bacteria. No soft rot will occur in plant tissues inoculated with water, no bacteria, or non-soft-rot bacteria. Different levels of virulence may be observed in plant tissues inoculated with comparable amounts of bacteria.

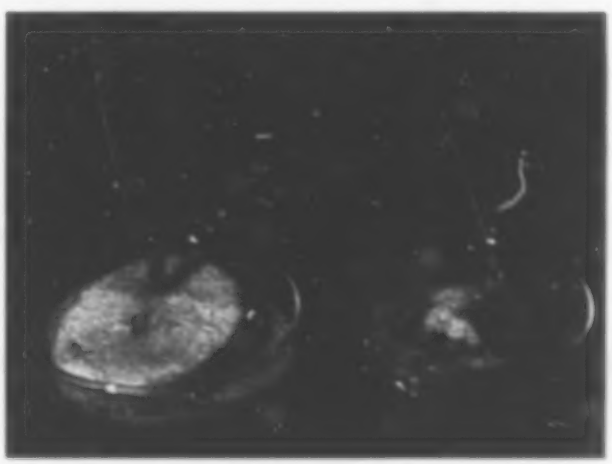

Figure 8H.3. *Spatula falls down when stuck in soft rotted potato (on right) but remains standing in uninoculated potato (on left).*

New soft rot bacterial isolates may be further identified as belonging to the genus *Erwinia* or *Pseudomo-*

*nas* by plating them on King's medium B to check for fluorescence. Refer to Chapter 6 and Experiment D. If they are fluorescent, they belong to the genus *Pseudomonas*; if they are not, they belong to the genus *Erwinia*.

PART B - Enzyme Assays: Maceration Test and Viscosity Test

***Purpose***

•To study the effect a preparation of the pectic enzyme polygalacturonase has on plant cells in the absence of bacteria.

•To study the relationship between a bacterium, its secreted enzyme, and the soft rot symptom in plants.

***Materials***

•24 hour cultures of *Erwinia carotovora* subsp. *carotovora* (Figure 8H.1) grown on nutrient agar (see Chapters 4, 7 and 8). Or soft rot bacterial isolates from rotting produce (cucumber, potato, green pepper). See end of experiment for method of isolating bacteria from plants.

•Inoculating loop.

•Cork borer, approximately size #9, sterile (wrap in foil and autoclave).

•Single edge razor blades, sterile (wrap in foil and autoclave).

•Indelible marker.

•Ethanol, 70%.

•Bleach, 10%.

•Tween 20® or a liquid detergent.

•Bunsen burners or alcohol lamps.

•Culture tubes of sodium polygalacturonate nutrient broth without glucose (recipe below).

•Sterile, empty, capped or plugged culture tubes (autoclave).

•0.2 micron filters and plastic syringe barrel and plunger (no needle) for sterilization of solutions.

•The pectic enzyme polygalacturonase (Sigma P3429).

•Sodium polygalacturonate (Sigma P1879), synonyms - sodium pectate, sodium polypectate.

•0.1M sodium acetate pH 4.5 (adjust pH with acetic acid).

•Several small beakers (50, 100 or 150 ml), 2 sterile (cap with aluminum foil and autoclave).

•Pasteur pipets, 6" sterile (wrap in aluminum foil and autoclave).

•Pipet bulbs.

•10-ml pipets, sterile (place in metal canister or wrap in aluminum foil and autoclave).

•Sterile toothpicks (place standing up in beaker, cap with foil and autoclave).

***Methods***

Read the safety information in chapter 4 on bleach, ethanol, sodium acetate, and acetic acid. All other chemicals pose little hazard, but always read the chemical label on the container.

*1) Maceration Test*

Prepare the liquid broth medium in tubes as follows:

Sodium polygalacturonate nutrient broth without glucose (SP broth)

| | |
|---|---|
| Distilled water | 500 ml |
| Beef extract | 1.5 g |
| Peptone | 2.5 g |
| Sodium polygalacturonate | 2.5 g |

On stir plate with metal stir bar, stir to dissolve all ingredients thoroughly in distilled water. Aliquot 10 ml each into culture tubes and cap loosely. Autoclave. Tighten caps and allow to cool before using. Recipe yields about 50 tubes. Adjust according to needs.

Using loop, inoculate at least two SP broth tubes with culture of *Erwinia carotovora* subsp. *carotovora* or known soft rot bacterium from your collection (Figure 8H.4a). Grow overnight at room temperature (with agitation on shaker, if available).

Prepare the 0.1M sodium acetate, pH 4.5. Place 50 ml of distilled water in a beaker and add 1.36 g of sodium acetate. Using a magnetic stir bar, stir until dissolved on a stir plate. With a pH meter, adjust pH to 4.5 by adding drops of acetic acid while stirring. Pour solution into 100 ml graduated cylinder and bring volume up to 100 ml by adding distilled water. Store solution in screw cap bottle on shelf.

The following day, prepare solution of pectic enzyme polygalacturonase as follows. Into sterile, empty, culture tube dispense 10 ml 0.1M sodium acetate pH 4.5 and to this add 0.2 ml enzyme. Tap tube to mix thoroughly. Dispense 5.1 ml (half) of enzyme solution into another sterile, empty, culture tube, cap both (Figure 8H.4b), and put in refrigerator to hold.

Check *E. c.* subsp. *carotovora* cultures for growth: one that is turbid (cloudy). This turbid broth culture will be split into thirds as detailed below (Figure 8H.4c).

Using sterile Pasteur pipet, remove one third of broth culture to sterile, empty, culture tube, cap it, and autoclave it to kill bacteria. At same time, autoclave one tube of polygalacturonase from refrigerator. (Check materials list to ensure that all sterile items have been autoclaved. Autoclave any materials lacking.)

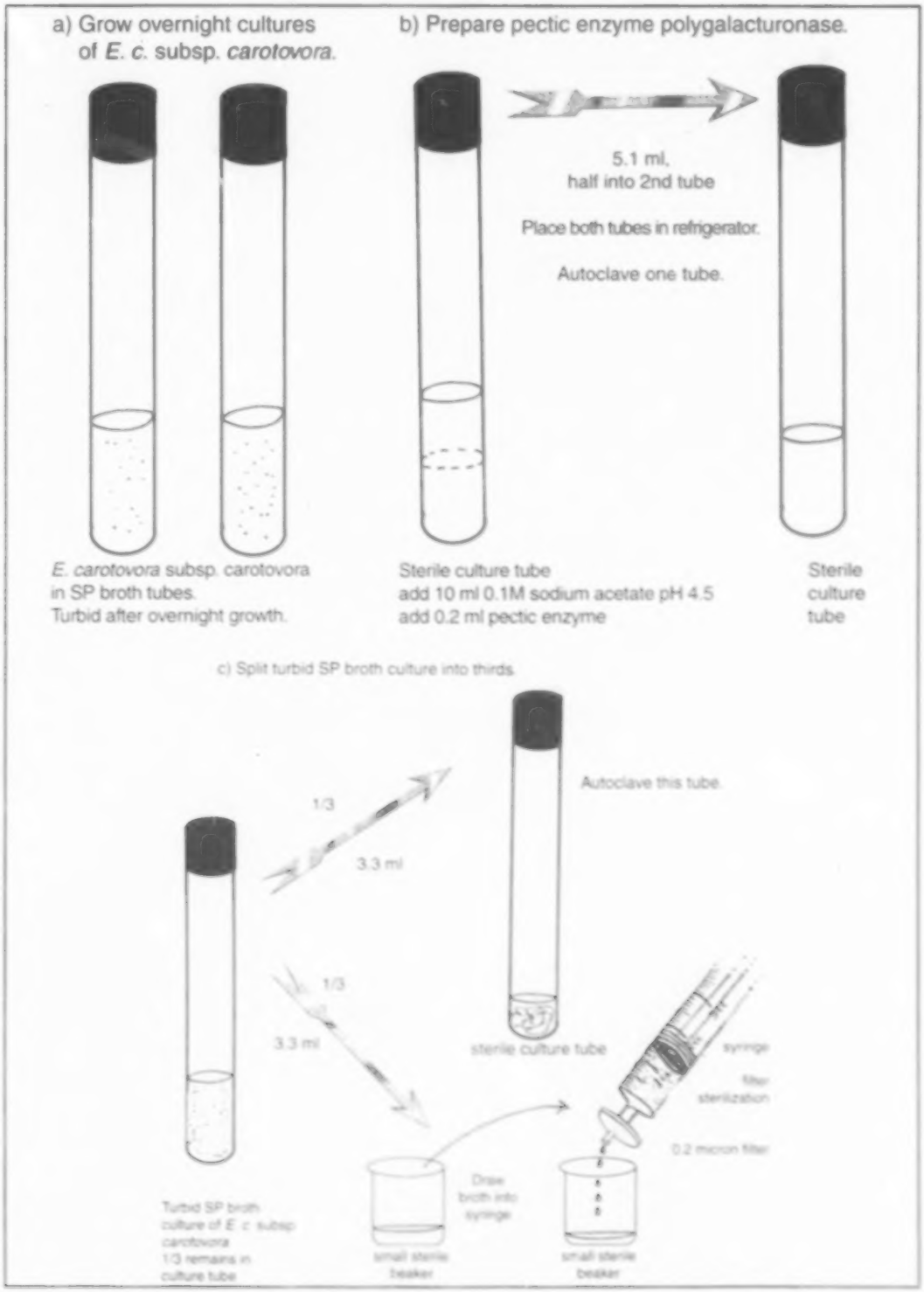
a) Grow overnight cultures of E. c. subsp. carotovora.
b) Prepare pectic enzyme polygalacturonase.
5.1 ml, half into 2nd tube
Place both tubes in refrigerator.
Autoclave one tube.
E. carotovora subsp. carotovora in SP broth tubes. Turbid after overnight growth.
Sterile culture tube add 10 ml 0.1M sodium acetate pH 4.5 add 0.2 ml pectic enzyme
Sterile culture tube
c) Split turbid SP broth culture into thirds.
1/3
3.3 ml
Autoclave this tube.
1/3
3.3 ml
sterile culture tube
syringe
filter sterilization
0.2 micron filter
Turbid SP broth culture of E. c. subsp. carotovora 1/3 remains in culture tube
Draw broth into syringe
small sterile beaker
small sterile beaker

Figure 8H.4. *Preparation of* Erwinia carotovora *sbsp.* carotovora *and polygalacturonase (pectic enzyme) for maceration assay.*

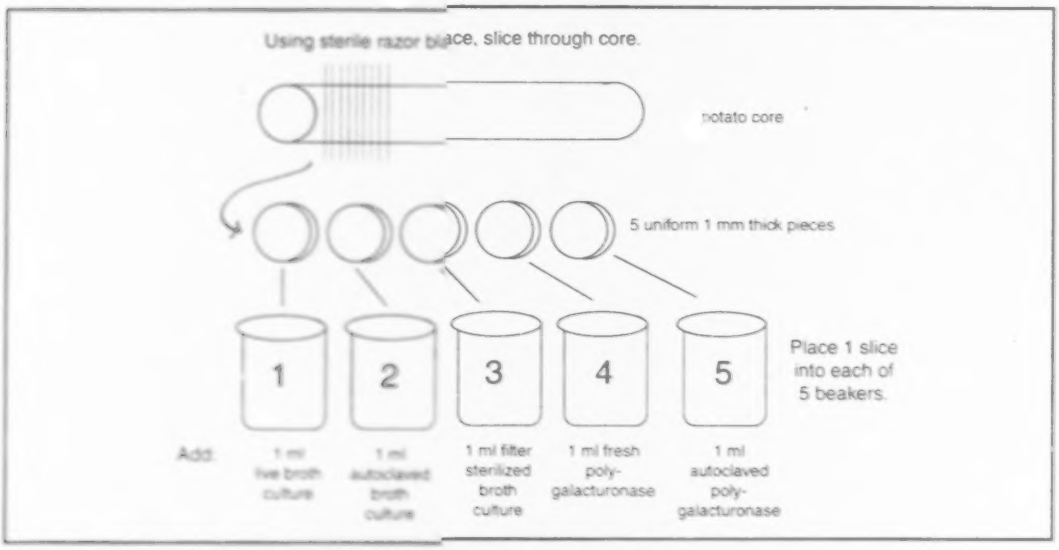


Figure 8H.5. *Preparation of tuber slices for maceration assay.*

Using sterile Pasteur pipet, remove another third of broth culture to small sterile beaker. Using syringe, suck up broth culture from beaker; screw 0.2 micron filter onto tip of syringe; and inject broth through filter into small, sterile beaker and recap with foil. Broth proceeds slowly through small pores (0.2 micron diameter) of filter as liquid is sterilized by removal of bacteria.

Leave final third of broth culture in its tube.

While things are autoclaving, surface sterilize a potato tuber: Immerse it in 10% bleach containing one drop of Tween 20 or another surfactant (liquid detergent). Allow to soak for 30 minutes. Transfer tuber to 70% ethanol and soak for 10 minutes. Remove tuber to clean surface and allow to dry. With sterile cork borer, remove several cylindrical cores from potato. Using sterile razor blades, cut 1-mm thick slices from potato cores - at least 10 discs. Place one slice each into five beakers and treat as follows:

1) Add 1 ml of live broth culture.
2) Add 1 ml of autoclaved broth culture.
3) Add 1 ml of filter sterilized broth culture.
4) Add 1 ml of fresh polygalacturonase (save remainder for viscosity test).
5) Add 1 ml of autoclaved polygalacturonase (save remainder for viscosity test).

See Figure 8H.5. Replicate above treatments once for each initial broth culture used. Leave treatments at room temperature and every 15 minutes probe slice with an

unused sterile toothpick to see if it breaks up easily, *i.e.* is macerated.

*2) Viscosity Test*

Prepare 2% sodium polygalacturonate solution as follows. Add 1 g of sodium polygalacturonate to 50 ml of 0.1M sodium acetate pH 4.5 and mix well on stir plate. Sodium polygalacturonate is a substrate for the enzyme polygalacturonase.

On barrels of several Pasteur pipets, make two marks spaced 2 cm apart (Figure 8H.6). Use indelible marker to draw lines around barrel. Pasteur pipets will be used to measure flow rate of liquids.

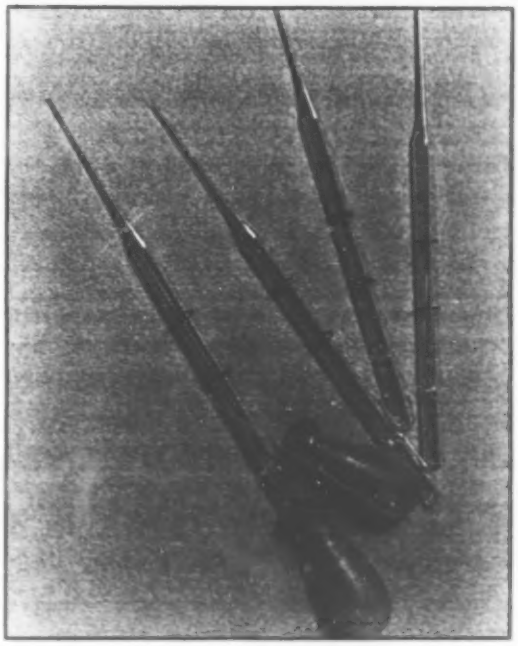

Figure 8H.6. *Pasteur pipets marked for the viscosity assay.*

When sodium polygalacturonate is thoroughly dissolved aliquot 2.5 ml into sterile culture tubes with sterile 10-ml pipet (yield 20 tubes). To determine flow rate or viscosity of solution through pipette prior to addition of enzyme, draw solution into a marked Pasteur pipet past top mark. Holding it above a beaker or liquid left in tube, gently remove bulb and measure time it takes for solution to drop from top mark to bottom mark. This is efflux time before treatment.

To half (10) of the tubes add 1 drop of nonautoclaved polygalacturonase and to other half (10) add 1 drop of autoclaved polygalacturonase. Label tubes accordingly. Tap or shake to mix and let stand for 10 minutes. Working in pairs with one person drawing liquid into pipet and another measuring flow rate, again draw solutions each into its own marked Pasteur pipet and measure efflux time after treatment.

***Results***

*1) Maceration Test*

Tissue maceration will occur in the potato slices treated with live bacteria, culture filtrate, and non-autoclaved polygalacturonase. No maceration will occur in the slices treated with autoclaved bacterial culture or autoclaved polygalacturonase.

*2) Viscosity Test*

The less viscous solution, the one in which polygalacturonate polymers have been cleaved by the enzyme, will travel through pipet faster. The sodium polygalacturonate

solution to which autoclaved polygalacturonase was added will travel slower down the pipet because the enzyme has been destroyed by autoclaving.

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***Isolation of Bacteria from Plant Tissues***

Soft rot is a distinct symptom readily recognized on produce in storage in the refrigerator or pantry. Cucumber, potato and green pepper are commonly attacked by these bacteria in post harvest storage. Sunken, soft, watery, off-color spots of rotting tissue can be observed.

Surface sterilize the vegetable by swabbing with 70% ethanol and allow to dry. With sterile technique, remove a small (4 x 4 mm) piece of tissue from the edge of the rotten lesion. The piece should consist most of healthy appearing tissue. Place the piece of tissue onto a nutrient agar plate. Repeat with 4 or 5 more pieces onto the same plate spacing them evenly apart. Or streak the tissue piece across the agar surface, allowing individual bacteria to be deposited on the plate. Incubate plate at room temperature for 24 to 48 hours. Streak out bacteria that grow from the tissue pieces to obtain single colonies as described in Chapter 8. Subculture the various distinct isolates obtained as seen by observing single, well separated colonies.

Using the soft rot bioassay above, determine which of the isolates obtained from the plant tissue are soft rot bacteria. Using King's medium B as described in Chapter 6 and in Experiment D, determine if the soft rot bacterium is a fluorescent Pseudomonas. Maintain any isolates of interest in your collection (see Chapter 7).

## I. The First Virus Discovered - Tobacco Mosaic Virus

***Introduction***

Tobacco mosaic virus (TMV) was the first virus discovered (Figure 8I.1). The virus is distributed worldwide and infects more than 150 genera of dicotyledonous plants. TMV is a very stable virus and can remain viable in dried, infected plant tissue for up to 50 years. For this reason, cigarette, cigar, pipe or chewing tobacco can serve as an inoculum source of this virus.

There are many distinct strains of TMV differing from each other in plant host range and the symptoms they cause. Symptoms on plants systemically infected include typical mosaic patterns on the leaves consisting of islands of light green and dark green tissues, stunting of leaves, flowers, fruits, and entire plants, and distortion of plant parts. Plants that have a hypersensitive reaction to the virus will develop small necrotic spots in response to the virus. The virus is localized in the necrotic tissue and the hypersensitive plants are resistant to further infection. The small necrotic spots that develop in response to virus inoculation are termed local lesions and form the basis for a bioassay for plant viruses.

***Purpose***

•To study a viral disease of plants by inoculating plants with TMV and observing for symptoms.
•To determine the titre of TMV in various sources of tobacco.

***Materials***

•TMV common strain from ATCC (see Chapter 5), cigarettes, cigars, pipe and chewing tobacco.
•Systemic host: Tomato plants var. New Yorker or a var. susceptible to TMV and/or petunia plants. Two or more plants for each source of TMV and two plants mock inoculated.
•Local lesion host: pinto bean plants. Six plants for each source of TMV and two plants mock inoculated.
•0.05M phosphate buffer, pH 7.0 or water.
•Carborundum or celite abrasives.
•Mortars and pestles thoroughly washed

Figure 8I.1. *Photograph of an electron microscope view of tobacco mosaic virus (TMV) rod-shaped particles (x 35,000).*

Top: Figure 8I.2. *Rub-inoculation method for plant viruses. Swab is dipped in liquid inoculum and rubbed onto young leaves.* Bottom: Figure 8I.3. *Piles of tobacco with mortars and pestles ready for grinding in phosphate buffer.*

and sterilized by either (1) baking for 3-4 hours at 110°C or (2) autoclaving or (3) soaking for 24 hours in 10% bleach, rinsing very well, and air-drying for 24 hours.

- Latex or rubber gloves.
- Cheesecloth pieces or cotton swabs.
- Several small beakers (one set of 5 for each source of TMV).
- 10-ml and 5-ml pipets.
- Pipet bulbs.

***Methods***

Read the safety information in Chapter 4 on bleach, carborundum, celite, and potassium phosphate dibasic and monobasic. All other chemicals pose little hazard, but always read the chemical label on the container.

Tomato or petunia plants should be about four weeks old and have at least two sets of true leaves. Such plants may be purchased as bedding plants or grown in a greenhouse or sunny windowsill. DO NOT USE VIRUS RESISTANT VARIETIES; they give local lesions or no symptoms.

Pinto bean plants should be about one week old, at the stage when the first two simple seed leaves are fully expanded. Grow these plants in a greenhouse or on a windowsill.

Prepare the phosphate buffer:

To 450 ml of water add 4.35 g potassium phosphate dibasic ($K_2HPO_4$) and 3.4 g potassium phosphate monobasic ($KH_2PO_4$). Mix to dissolve. Bring volume up to 500 ml with water. Store in refrigerator.

*1) Systemic Infection*

As controls, rub the leaves of a separate set of tomato or petunia plants using only water or buffer plus a dash of abrasive. Dip cheesecloth or swab into liquid and rub the liquid onto the upper leaf surface while supporting the leaf from below with other hand (Figure 8I.2). Gently rinse inoculated leaves in running water. Label the plants as water or buffer control, and include plant name and date of inoculation. Set these plants aside where they will be grown to avoid contaminating them with TMV-inoculated plants.

Unravel the cigarettes and cigars. Place the tobaccos in separate piles (Figure 8I.3). Thoroughly wash hands with soap and water after handling the tobacco to prevent inadvertent contamination of plants with TMV. Use two to four systemic host plants for each source of tobacco.

Grind the tobacco from each source in a separate mortar adding 9 ml phosphate buffer or water for each gram of tobacco. Add a small quantity of either carborundum or celite abrasive to help break the tissue to release the virus particles. The abrasive also helps break the epidermal cells on the inoculated plants and this is thought to favor virus infection. Avoid inhaling the carborundum or celite dust. Wear gloves to prevent skin irritation from the abrasive and to prevent contamination of hands with TMV.

Follow ATCC directions for using their common strain of TMV.

Dip a cheesecloth or swab into the liquid in one of the mortars and gently rub the liquid onto the upper surface of the leaves of the systemic host plants. Change gloves or thoroughly wash hands and repeat this procedure for the other sources of TMV using fresh cheesecloth or swabs.

Label each plant with the source of inoculum, plant name, and date of inoculation.

Gently rinse inoculated leaves in running water. For local lesion bioassay, save liquid from each ground TMV source by pouring it into a labeled beaker. Squeeze liquid from tobacco, if necessary.

Place all the plants in a greenhouse or sunny window and after one week begin observing the new growth daily. Symptoms of TMV infection can be very subtle on tomato. Comparison with water- or buffer-inoculated plants will allow the observation of virus-induced symptoms.

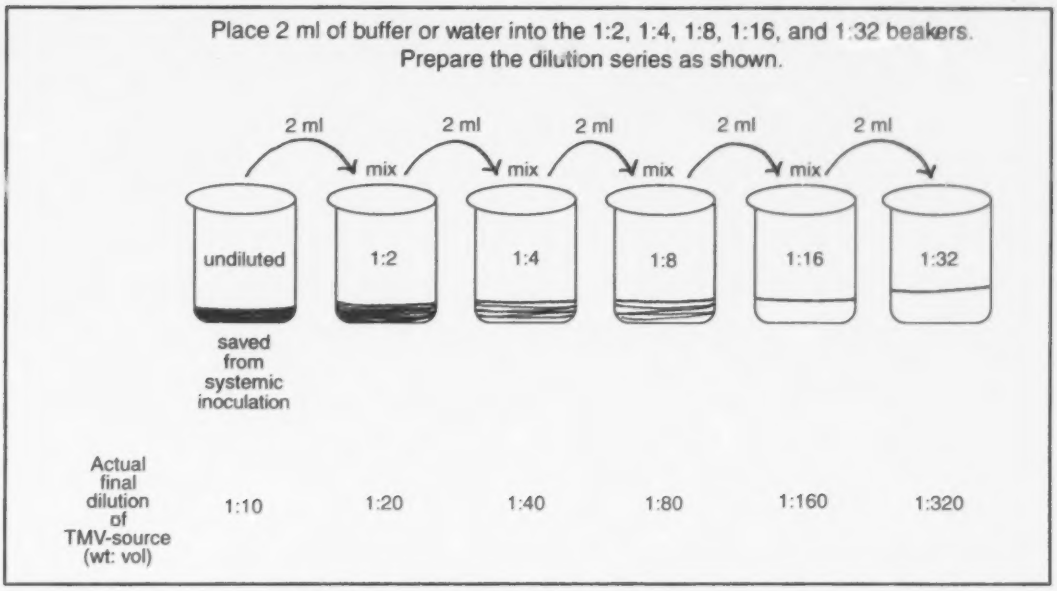


Figure 8I.4. *Preparation of dilution series for local lesion bioassay.*

*2) Local Lesion Bioassay*

Run a bioassay on the pinto bean plants to determine the relative titer of TMV in each source of tobacco.

Thoroughly wash hands, put on a clean pair of gloves, and mock inoculate one leaf on each of 2 pinto bean plants with either water or buffer plus abrasive to serve as controls. Gently rinse the inoculated leaves in running water.

Using each of the inocula from the various sources of tobacco, prepare a dilution series of 1:2, 1:4, 1:8, 1:16 and 1:32 (Figure 8I.4). Label a set of 5 small beakers for each inoculum source and the dilutions. With pipet add 2 ml of either buffer or water to each beaker. Use the same liquid (buffer or water) that the inoculum was initially prepared in. Add a small amount of abrasive to each beaker to make certain it is not diluted out as the dilutions are made. Prepare the dilution series for each inoculum source: With a clean pipet, remove 2 ml of liquid from the inoculum reserved from " systemic infection" and place it into its respective 1:2 dilution beaker. Pipet up and down to rinse the inside of the pipet and thoroughly mix the 1:2 dilution. Remove 2 ml from the 1:2 dilution. Add to the respective 1:4 dilution beaker, pipet up and down to rinse pipet and mix solution. Remove 2 ml from this and add to the respective 1:8 beaker. Continue in the same manner to prepare the 1:8, 1:16 and 1:32 dilutions. Prepare a dilution series using each source of tobacco used in "systemic infection." For each use a clean pipet.

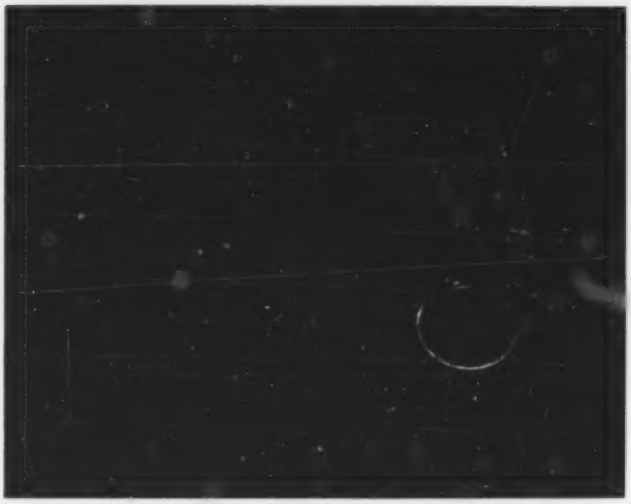

Figure 81.5. *Symptoms of tobacco mosaic virus (TMV) infection. Healthy plant on left, infected plant on right.*

For each source of tobacco, with the undiluted inoculum, inoculate six pinto bean plants as follows. Using leaf midrib as dividing line, rub half of one leaf on each of two pinto bean plants. Rub the other half of the same leaf with the 1:2 dilution. On 2 more pinto bean plants, inoculate half of one leaf on each plant with the 1:4 dilution and the other half with the 1:8 dilution. On a final set of two pinto bean plants, inoculate half of one leaf on each plant with the 1:16 dilution and the other half with the 1:32 dilution. For each source of tobacco, use a fresh cotton swab or piece of cheesecloth and change gloves or wash hands after rubbing each dilution. Leave the other leaf on each of the pinto bean plants uninoculated. Label each plant with the source of tobacco, dilutions on half-leaves, and the date.

Gently rinse the inoculated leaves in running water.

Place the plants in a greenhouse or sunny window and observe them daily.

Count the number of local lesions on each half leaf to determine the relative titer of virus in each dilution. Theoretically, one local lesion corresponds to approximately 10,000 to 100,000 TMV virus particles.

***Results***

Systemic mosaic symptoms will show on the developing leaves of the tomato or petunia plants. The symptoms may be very subtle. On the youngest leaves look for leaf distortion, cupping, and faint mosaic symptoms (Figure 81.5). Are there differences in symptoms from each of the tobacco sources? This may result from distinct strains of the virus being present in the different tobaccos compared to the common strain obtained from ATCC.

Small necrotic spots termed local lesions should develop on the pinto bean leaves. To determine the relative titre of the virus in each source of tobacco, count the local lesions on each half leaf. Fewer local lesions will occur as inoculum is diluted. There

may be wide variation in local lesion numbers from the same source of inoculum depending on the pressure applied when rubbing the leaf during inoculation and on the leaf itself. Which source of tobacco is the richest source of virus?

On all inoculated plants, damage may occur to leaves from the rubbing. Compare mock-inoculated leaves with virus-inoculated leaves.

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*NOTE: For future experimentation, TMV can readily be saved from tomato plants showing symptoms of systemic infection or from individual local lesions. Simply remove the most symptomatic leaves or cut out isolated local lesions and dry them over calcium carbonate or silica gel or press whole leaves in a plant press or hang them in a paper bag as you would dry herbs. Once dry, place the leaves or individual local lesions in an air-tight vial or jar, label, and freeze for future use. In addition, local lesions can be cut out of fresh leaves, ground in buffer, and inoculated onto a systemic host as a way of obtaining nearly pure strains of viruses.*

## J. Plant Tumors Are Genetically Engineered by *Agrobacterium tumefaciens*

***Introduction***

*Agrobacterium tumefaciens* causes the disease known as crown gall. Crown gall is considered one of the most important economic diseases of plants in the United States. It is known to occur worldwide. Virulent strains of *A. tumefaciens* carry a large plasmid that contains several genes, among them plant tumor-inducing genes. Plasmids are independently replicating molecules of extra-chromosomal DNA; many are circular and double-stranded. Plasmids carry genes separate from the immense single chromosome found in a bacterium. In *A. tumefaciens*, the large plasmid carrying the tumor-inducing (Ti) genes is termed the Ti plasmid. The tumor-inducing genes lie on regions of DNA in the Ti plasmid that are transferred into plant cells following infection by the bacterium. The transferred DNA (T-DNA) is incorporated into the chromosomes of infected plant cells. The genes in the transferred region code for auxin and cytokinin production, hormones that promote plant cell growth. Continued, uncontrolled cell growth leads to the formation of galls. The pathogenic bacteria are found in the intercellular spaces of the outer tissues of galls. Only the T-DNA is found within the plant cells - integrated into the plant's chromosomal DNA. The T-DNA region of the Ti plasmid and the plasmid itself have been engineered in the laboratory to deliver desirable genes into plants instead of the cytokinin and auxin genes. For example, tobacco plants have been engineered using *Agrobacterium*-mediated transformation with viral genes to confer resistance to TMV, tobacco mosaic virus.

There are many strains of *A. tumefaciens,* found in nature, each carrying one or more types of plasmids. Strain C58 is one that, after continous culture at 37° C, loses the Ti plasmid and with it the ability to cause galls on plants. This characteristic was instrumental in proving the role of the Ti plasmid in pathogenesis. Another species of *Agrobacterium, A. rhizogenes*, carries a root-inducing plasmid. Tumors caused by this bacterium are called hairy root tumors because of the proliferation of many roots at the site of infection.

PART A - Crown Gall on Plants

***Purpose***

•To demonstrate the symptoms of crown gall on plants.

•To determine the effect of Ti plasmid containing strains versus non-Ti plasmid containing strains on disease development.

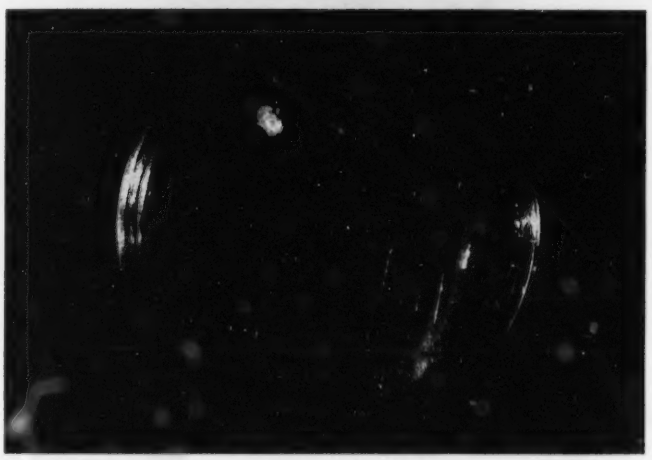
***Materials***

•Young *Kalanchoe daigremontiana* (Kalanchoe, devil's backbone, mother-of-thousands) (Figure 8J.1), tomato or sunflower plants about 4 to 6 inches (10 to 15 cm) tall. No plant is infected by more than 81% of the pathogenic strains.

•Nutrient agar plates (see Chapter 6).

•48-hour cultures, grown at 25° C or room temperature, of both *Agrobacterium tumefaciens* strains A348 (wild type, causes galls) and A136 (heat cured, does not cause galls) grown on nutrient agar (Figure 8J.2), one plate each per lab group (see Chapters 5 and 7). Other strains of *A. tumefaciens* are available from Carolina Biological Supply and Ward's Scientific. (These may all cause galls on plants.)

•Sterile toothpicks (place standing in beaker, cap with foil, and autoclave).

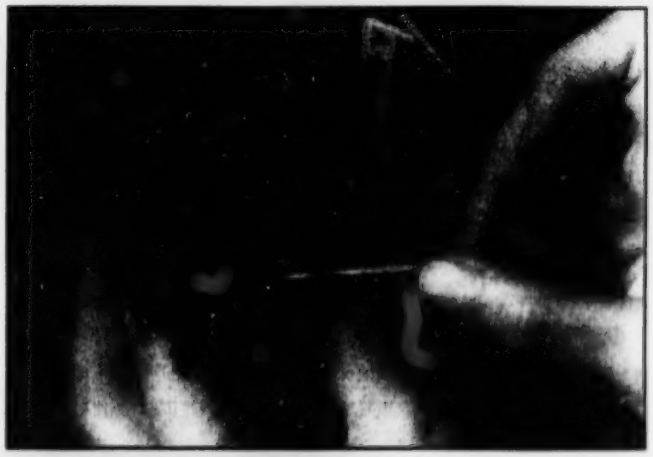


Top: Figure 8J.1. *A young Kalanchoe plant suitable for* Agrobacterium *inoculation of leaves and stems*. Bottom: Figure 8J.2. *24-48 hour culture of* Agrobacterium tumefaciens *on nutrient agar.*

***Methods***

Grow or purchase host plants. Grow Kalanchoe, tomato or sunflower plants in flats or pots of sterile soil and fertilize with a commercial nutrient solution every three weeks. When optimum light and temperatures are available, inoculations can be made 14 days from seeding the tomato and sunflower. Tomato or Kalanchoe plants may be purchased from a local greenhouse or garden center. *Kalanchoe daigremontiana*, may be available as a house plant; and the plantlets that grow on leaf margins may be propagated and used for inoculations.

Plants are ready for inoculation when they are 4 to 6 inches (10 to 15 cm) tall and have four to six leaves (Figure 8J.1). When plants are close to proper size for inoculation, grow pure cultures of *A. tumefaciens* strains A348 and A136 as described in Chapter



Top: Figure 8J.3. *Inoculation of stem of Kalanchoe.* Middle: Figure 8J.4. *Inoculation of leaves of Kalanchoe.* Bottom: Figure 8J.5. *Galls on Kalanchoe leaves and stems caused by* Agrobacterium tumefaciens.

7 and subculture twice per week, if necessary. Bacteria kept by subculturing may have reduced virulence, therefore plan to begin cultures at about the same time as plants are ready. Two days prior to inoculation of plants, streak out the bacteria onto nutrient agar plates and grow at room temperature.

With tip of sterile toothpick, pick up cells from an isolated colony of *A. tumefaciens* strain A136.

Gently stab cells into plant stem just below a leaf petiole (Figure 8J.3) making certain the cells remain inside the stem. Repeat for other species of plants being grown. For Kalanchoe, also inoculate a succulent leaf by scraping bacterial cells into the upper surface (Figure 8J.4)

Repeat these inoculation procedures using *A. tumefaciens* strain A348.

Leave at least one plant of each species uninoculated and/or one stabbed with a sterile toothpick without bacteria (mock inoculated) as controls.

Label each plant with the student's name, the isolate name, and the date of inoculation. Pot labels, string tags, or tape work well for labeling.

After inoculations, keep plants in high humidity for 1 to 2 days. Enclose plants in clear plastic bags or frequently mist them with sterile water in a hand pump sprayer. Remove the plants from the humid environment, and place plants in a greenhouse or a sunny window to grow. Repot the plants into larger pots as needed.

Observe once a week for the formation of galls and rate gall formation after 4 weeks (Figure 8J.5). Bacterial-induced galls will continue enlarging as the plant grows. When plants are inoculated with a very small number of bacterial cells, galls may take longer to develop.

***Results***

Galls should form at the inoculation sites on plants inoculated with strain A348. No galls will form on plants inoculated with strain A136, uninoculated, or mock inoculated. Galls will appear as follows:

| Plant | Inoculation Site | Gall Morphology |
|---|---|---|
| Kalanchoe | stem | Tumor with peripheral roots |
| Kalanchoe | leaf | Undifferentiated tumor |
| Tomato | stem | Undifferentiated tumor |
| Sunflower | stem | Undifferentiated tumor |

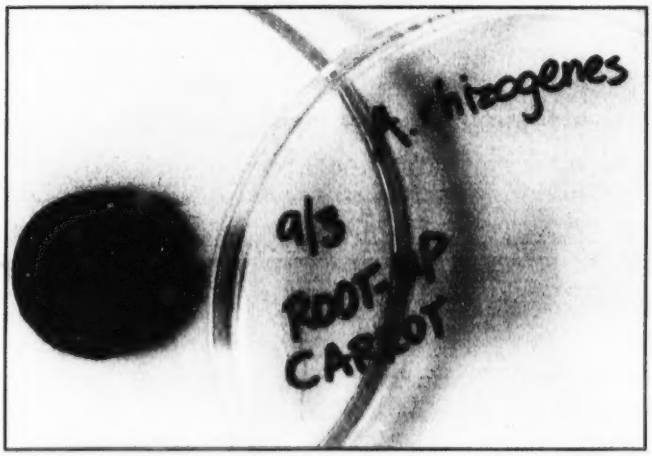
PART B - Shoots or Roots: Tumefaciens or Rhizogenes

***Purpose***

•To demonstrate the difference between *A. tumefaciens* and *A. rhizogenes* tumors.
•To illustrate that plant parts may retain their inherent shoot and root signals at the cellular level.

***Materials***

•Carrots.
•Sterile, filter paper-lined petri plates (Figure 6.1A and B, Chapter 6) and sterile 4-ml water blanks (see Chapter 6) OR water agar plates (see Chapter 6).
•20% bleach.
•Large forceps (to handle carrots).
•3 large beakers half full of sterile water.
•Knife.
•Nutrient agar plates (see chapter 6).
•48-hour cultures of *A. tumefaciens* strain A348 (Figure 8J.2) (or any gall-inducing strain), strain A136 and *A. rhizogenes* growing on nutrient agar plates (see Chapters 5 and 7).




Top: Figure 8J.6. *Undifferentiated galls on carrot disc inoculated with* Agrobacterium tumefaciens. Bottom: Figure 8J.7. *Rooty galls on root-up carrot disc inoculated with* Agrobacterium rhizogenes.

•Sterile toothpicks (place standing in beaker, cap with foil, and autoclave).

***Methods***

Read the safety information in chapter 4 on bleach and ethanol. All other chemicals pose little hazard, but always read the chemical label on the container.

Remove leaves from carrots, if necessary. Scrub carrots with detergent and rinse with tap water. Soak in 20% bleach for 5 minutes. Rinse three times in sterile water using the three beakers sequentially. Allow carrots to remain in the third rinse.

Surface sterilize a work surface by wiping it with 70% ethanol or 10% bleach. Also wipe a knife to surface sterilize it. On this surface, remove carrots from rinse and cut carrot discs 1/4 inch (1/2 cm) thick. Keep track of the shoot or upper side of the slices and the root or lower side of the slices.

With surface-sterilized forceps, place carrot discs, one per plate, in plates lined with paper or in water agar plates. Place half the discs shoot side up and half the discs root side up. Label plates accordingly. Prepare four sets of shoot-up and root-up carrot discs per lab group.

Add about 4 ml sterile water to the paper-lined plates to moisten the paper. Avoid pouring water on the carrot disc.

Inoculate one set of carrot discs with *A. tumefaciens* A348, one set with *A.*

*tumefaciens* A136, and one set with *A. rhizogenes*. Leave one set uninoculated. With a sterile toothpick, pick up bacterial cells from a single colony and smear the cells onto the carrot disc's upper surface. Label the plates with the name of the bacterial isolate, the date and your name.

Incubate the carrot disc plates at room temperature for three or more weeks. Add more sterile water to the paper to maintain moisture as needed. Observe the discs for the formation of galls. Some carrot discs may decay or rot from air-borne contaminating microorganisms during the long incubation period.

***Results***

No galls will form on the uninoculated discs and the discs inoculated with *A. tumefaciens* A136. Undifferentiated galls will form on both shoot-up and root-up discs inoculated with *A. tumefaciens* A348 (Figure 8J.6). Undifferentiated galls will form on carrot discs inoculated with *A. rhizogenes* but after two or three weeks the root-up disc will have tiny roots growing out among the galls (Figure 8J.7). (Roots that grow from the sides of the carrot discs are normal roots.)

PART C - Heat Curing the Ti Plasmid

***Purpose***

To cure the Ti plasmid from strain C58.

***Materials***

- Plants as described in Part A.
- Nutrient agar plates (see Chapter 6).
- 48-hour cultures of *Agrobacterium tumefaciens* strain C58 grown on nutrient agar (see Chapters 5 and 7).
- An incubator set at 37°C.
- Transfer loop.
- Alcohol lamp or bunsen burner.

***Methods***

Grow plants for inoculation as described in Part A. While plants are growing, heat-cure strain C58 as described below. Sterile technique, knowledge of colony morphology of C58, and the use of single, isolated colonies are very important. Refer to Chapter 7.

Transfer a single, isolated colony of C58, growing in pure culture, to nutrient agar, streaking to obtain isolated single colonies. Repeat with two or more plates. Examine colonies for color, appearance and edges and make note of these. Use a dissecting microscope with transmitted light, if available (Chapter 7).

Incubate at 37°C. Every three days transfer a single colony from each plate to a new plate, streaking to obtain isolated single colonies and incubate at 37°C. After the transfer on Day 15, incubate the bacteria at room temperature (21°C) for 48 hours. These transfers will be used for plant inoculation.

Maintain a set of cultures in a similar manner at room temperature.

Choose single colonies from the 37°C and the 21°C treatments for plant inoculations.

Repeat plant inoculation procedures as described in Part A using the heat treated cultures and the room temperature cultures. Label plants with name of isolate used for inoculation, date and your name.

Grow plants and observe for galls as described in Part A.

***Results***

Isolates of strain C58 may lose gall-forming ability after growth at 37°C. Loss of the Ti plasmid may not occur in 100% of the bacterial population. Careful tracking of single bacterial colonies during heat treatment will improve success rate. A reduction in size or rate of gall formation may be found when only a small percentage of the bacterial population in the single colony used for inoculation retains the Ti plasmid.

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Figure 8A.18: Kenaga, Clare B. 1970. *Principles of Phytopathology.* Balt Publishers, Lafayette, IN. In order: Figures 4.15B, 4.18A, 4.18B, 4.15D, 4.16B, 4.17B.
Figure 8A.19: Department of Plant Pathology, Cornell University, Ithaca, NY.
Figure 8A.20: Department of Plant Pathology, Cornell University, Ithaca, NY.
Figure 8B.1: Thomas A. Zitter, Department of Plant Pathology, Cornell University, Ithaca, NY.
Figure 8B.2: Department of Plant Pathology, Cornell University, Ithaca, NY.
Figure 8B.3 (a): Christopher M. Becker, American Cyanamid, Romulus, NY.
Figure 8B.3 (b): Linda Hanson, Department of Plant Pathology, Cornell University, Ithaca, NY.
Figure 8B.4: Juliet E. Carroll, Department of Plant Pathology, Cornell University, Ithaca, NY.
Figure 8C.1: Kenaga, Clare B. 1970. *Principles of Phytopathology.* Balt Publishers, Lafayette, IN. Figure 2.4.
Figure 8C.2: Department of Plant Pathology, Cornell University, Ithaca, NY.
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Figure 8D.1: Mai, William F. & Lyon, Howard H. 1975. *Pictorial Key to the Genera of Plant Parasitic Nematodes.* Cornell University Press, Ithaca, NY. Plate 44 after s'Jacob 1959, Nematologica.
Figure 8D.2: Peter Mullin, Department of Plant Pathology, Cornell University, Ithaca, NY.
Figure 8D.3: Department of Plant Pathology, Cornell University, Ithaca, NY.
Figure 8D.4: Peter Mullin, Department of Plant Pathology, Cornell University, Ithaca, NY.
Figure 8E.1: Department of Plant Pathology, Cornell University, Ithaca, NY.
Figure 8E.2: Department of Plant Pathology, Cornell University, Ithaca, NY.
Figure 8E.3 (a): Christopher M. Becker, American Cyanamid, Romulus, NY.
Figure 8E.3 (b): Gary A. Harman, Department of Horticultural Sciences, New York Agricultural Experiment Station, Cornell University, Geneva, NY.
Figure 8E.4: Department of Plant Pathology, Cornell University, Ithaca, NY.
Figure 8E.5: Department of Plant Pathology, Cornell University, Ithaca, NY.
Figure 8F.1: Juliet E. Carroll, Department of Plant Pathology, Cornell University, Ithaca, NY.
Figure 8F.2: Juliet E. Carroll, Department of Plant Pathology, Cornell University, Ithaca, NY.
Figure 8F.3: Department of Plant Pathology, Cornell University, Ithaca, NY.
Figure 8F.4: Department of Plant Pathology, Cornell University, Ithaca, NY.
Figure 8F.5: Illustration by Nina Shishkoff, Figure 14. In: *Know Your Tree Diseases* by Juliet E. Carroll, 4H Bulletin J-116, Cornell Cooperative Extension, Ithaca, NY.
Figure 8G.1: Thomas A. Zitter, Department of Plant Pathology, Cornell University, Ithaca, NY.
Figure 8G.2: Department of Plant Pathology, Cornell University, Ithaca, NY.

Figure 8G.3: Department of Plant Pathology, Cornell University, Ithaca, NY.
Figure 8G.4: Department of Plant Pathology, Cornell University, Ithaca, NY.
Figure 8H.1: Department of Plant Pathology, Cornell University, Ithaca, NY.
Figure 8H.2: Department of Plant Pathology, Cornell University, Ithaca, NY.
Figure 8H.3: Alan Collmer, Department of Plant Pathology, Cornell University, Ithaca, NY.
Figure 8H.4: Juliet E. Carroll, Department of Plant Pathology, Cornell University, Ithaca, NY.
Figure 8H.5: Juliet E. Carroll, Department of Plant Pathology, Cornell University, Ithaca, NY.
Figure 8H.6: Department of Plant Pathology, Cornell University, Ithaca, NY.
Figure 8I.1: Juliet E. Carroll, Department of Plant Pathology, Cornell University, Ithaca, NY.
Figure 8I.2: Illustration by R.J. Goetz.
Figure 8I.3: Department of Plant Pathology, Cornell University, Ithaca, NY.
Figure 8I.4: Juliet E. Carroll, Department of Plant Pathology, Cornell University, Ithaca, NY.
Figure 8I.5: Department of Plant Pathology, Cornell University, Ithaca, NY.
Figure 8J.1: Department of Plant Pathology, Cornell University, Ithaca, NY.
Figure 8J.2: Department of Plant Pathology, Cornell University, Ithaca, NY.
Figure 8J.3: Department of Plant Pathology, Cornell University, Ithaca, NY.
Figure 8J.4: Department of Plant Pathology, Cornell University, Ithaca, NY.
Figure 8J.5: Department of Plant Pathology, Cornell University, Ithaca, NY.
Figure 8J.6: Department of Plant Pathology, Cornell University, Ithaca, NY.
Figure 8J.7: Department of Plant Pathology, Cornell University, Ithaca, NY.